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AD_____

Award Number: DAMD17-96-1-6038

TITLE: Growth Suppression and Therapy Sensitization of Breast Cancer

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CONTRACTING ORGANIZATION: Sidney Kimmel Cancer Center
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REPORT DATE: July 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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Nancy Chan Reuter 01/16/2002 _____

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Final (1 Jul 96 - 30 Jun 01)

4. TITLE AND SUBTITLE
Growth Suppression and Therapy Sensitization of Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6038

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The goal of this project is to provide a rationale and pre-clinical evaluation of p53-based approaches to growth suppression and therapy sensitization of breast cancer, including combination approaches in which p53 gene therapy is combined with traditional chemotherapy or with inhibitors of DNA repair. We have observed that restoration of p53 activity in tumor cells sensitizes them to a variety of DNA damaging treatments, including doxorubicin, a standard breast cancer therapeutic. An important determinant of p53-mediated apoptosis is the level of endogenous DNA damage, and this is increased by treatment of cells with inhibitors of DNA repair. We have also observed that disruption of the Jun Kinase pathway inhibits DNA repair, increases the sensitivity of tumor cells to DNA damage and to p53-mediated apoptosis through the bax pathway. Intratumoral administration of a replication-impaired adenovirus encoding wild-type p53 (Adp53) suppresses the growth of established subcutaneous tumors of human breast cancer cells in nude mice, and this suppression is enhanced in the presence of doxorubicin. Systemic administration of Adp53 in combination with doxorubicin results in a significant reduction in the incidence of lung metastases, using an orthotopic model in nude mice of human metastatic breast cancer.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
Breast Cancer

15. NUMBER OF PAGES
134

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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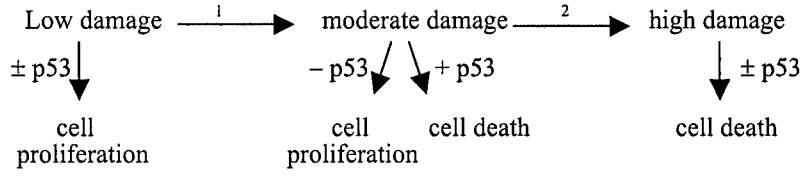
INTRODUCTION

The subject of this research was the mechanism of p53-mediated tumor suppression and therapy sensitization, and the potential of p53 gene transfer, alone or in combination with conventional therapy or other gene-based approaches, to treat breast cancer, a disease in which p53 abnormalities occur in about 25-40% of cases. Inactivation of the p53-mediated pathway of apoptosis through loss of wild-type p53 expression or other components of the pathway is probably fundamental to the mechanism of cancer. Because the pathway may be a particularly vulnerable step in cancer cell metabolism, but may have negligible consequences for normal cells, it provides an opportunity for developing non-toxic therapeutic interventions selective for cancer cells. The study was based on evidence from our own laboratory and those of others that p53 gene transfer dramatically improved the response of cancer cells to traditional therapies. This property of p53 has now been exploited therapeutically and is in an advanced stage of clinical development, with several Phase I and II trials of p53-based approaches for various cancers, including breast cancer, in progress or completed.

The purpose of our study was to examine the efficacy of p53-based approaches to breast cancer, including combination approaches that used p53 together with conventional DNA damaging therapies such as doxorubicin (adriamycin) and cisplatin, and/or inhibitors of DNA repair. The study focused on the involvement of p53 in the cellular DNA damage response, and proposed that by exploiting the role of p53 in DNA damage recognition and damage-induced apoptosis we could potentiate the therapeutic efficacy of p53-based therapies. The underlying hypothesis we set out to test is illustrated in the scheme below:



We hypothesized that the level of DNA damage would be a critical determinant of p53-mediated apoptosis. Thus, at low levels of endogenous DNA damage (DNA strand breaks, abnormal DNA structures such as insertion-deletion loops) characteristic of tumor cells with unstable genomes, p53 expression would be compatible with cell survival. At moderate levels of DNA damage (step 1, achieved through treatment with DNA damaging chemotherapeutic drugs or through inhibition of DNA repair) p53 expression becomes incompatible with survival and apoptosis is induced. At higher levels of DNA damage (step 2, achieved by a combination of DNA damage induction and inhibition of DNA repair), cells will die irrespective of p53 status. Based on earlier work from other laboratories implicating the AP-1 transcription factor in DNA repair and in the cellular response to DNA damaging drugs such as cisplatin (1, 2), we hypothesized that inhibition of Jun Kinase pathway would promote p53-mediated apoptosis and contribute to the therapy response, Because this pathway targets AP-1 regulated genes.

In order to test this hypothesis and to better understand the involvement of p53 in the therapy response we proposed to generalize our initial observations to other breast cancer cell lines of defined p53 status and to a variety of drugs acting through different mechanisms. We proposed mechanistic studies to elucidate the molecular basis for therapy sensitization. In addition, we

proposed a novel strategy, in which p53 gene replacement would be used in combination with one of two approaches to inhibit AP-1 regulated gene expression: through modification with a non-phosphorylatable mutant of c-Jun (mutant Jun) to inhibit c-Jun downstream targets (including AP-1 regulated genes), or by treatment with AP-1 inhibitory retinoids. Finally, in order to evaluate the clinical potential of p53 combination approaches, we proposed to use several nude mouse models for human breast cancer.

BODY OF REPORT

Described below are the research accomplishments associated with each task outlined in the Statement of Work.

TECHNICAL OBJECTIVE I: *IN VITRO* DRUG AND RADIATION SENSITIVITY STUDIES.

Task 1: Evaluate the sensitivity of various breast carcinoma cells lines to various drugs and radiation. In order to understand the scope of the p53-mediated therapy sensitization effect breast cancer cell lines of varying p53 status were assayed for sensitivity to ectopic wild-type p53 expression in the presence and absence of various treatments. Cell lines were chosen so as to represent p53 mutants with dominant-negative effects over wild-type p53 (codon 280 mutation (3)), and mutants for which dominant-negative effects have not been described (codons 194, 266). Because many of the p53 mutants in human cancers may be dominant-negative mutants, such an analysis would have relevance to the clinical application of p53-based therapies for a wide range of cancers. The cell lines analyzed are listed in Table 1 and the treatments are listed in Table 2.

Table 1
p53 status of breast cancer cell lines

Cell line	*P53 status*	*Dominant-negative mutation*
T47D	Mutant: codon 194 (CTT (leu) → TTT (phe))[a]	no
MCF7	Wild-type[b]	
MDA MB 231	Mutant: codon 280 (AGA (arg) → AAA (lys))[a]	Yes[d]
MDA MB 435	Mutant: codon 266 (GGA (gly) → GAA (glu))[c]	no

a) see http://www.iarc.fr/p53/; b) Cai, Z., et al (1997) Oncogene 15: 2817; c) Lesoon-wood, et al (1994) Hum Gene Therapy 6: 395; d) Brachman et al (1996) Proc Natl Acad Sci 93: 4091.

Table 2
Mechanism of action of various chemotherapeutic drugs

Chemotherapeutic drug	*Mechanism of action*
cisplatin	DNA damage (primarily bifunctional adducts)
Doxorubicin (adriamycin)	Multiple modes, including DNA damage (double strand breaks)
5-fluorouracil	Multiple modes, including DNA damage
taxotere	Microtubule inhibitor (antimitotic)

In order to assay how ectopic expression of p53 affects the response of these cell lines to chemotherapeutic drugs, we used a p53 adenovirus (Adp53) and control adenovirus (expressing either luciferase (AdLuc) or ß-galactosidase (Ad-ßgal)). The viruses were supplied by Introgen Therapeutics, Inc. Adenovirus-mediated gene transfer was chosen for its unparalled gene transfer efficiency, and for its promise as a clinical tool to achieve gene transfer in vivo. The in vitro assays were carried out in a 96 well format, as we have previously described (4). Briefly, cells were plated in 24 well plates on day 1 such that their density would be about 70% on day 2, a density where we observed optimal viral infection efficiencies. On day 2, cells were treated with sufficient Adp53 to achieve a 30% - 50% reduction in cell viability at 5 days (as determined in pilot assays). For the

cell lines we used, this ranged from 100 – 200 pfu of virus for 3-4 hours. After treatment with virus, cells were replated in 96 well plates at 1000 cells per well, a density low enough to ensure that control (non-drug treated) wells would remain subconfluent during the subsequent 5 days of incubation. On day 3, successive row of cells (quadruplicate wells in each row) were treated with increasing concentrations of drug as follows: cisplatin (0-50 μM, 1 hour), doxorubicin (0-1 μM, 4 hours), 5-fluorouracil (0-20 μM, 1 hr), taxotere (0-0.1 μM, 1 hr). After treatment, medium was replaced, and cells were allowed to incubate an additional 5 days. Viability was determined by the MTS assay, which measures the bioconversion of a formazan compound to a colored derivative, detectable by measuring absorbance at 540 nm. For each vector treatment, the viability of drug treated rows was expressed as a percentage of the viability in non-drug treated rows. Thus an additive effect of Adp53 and drug would result in AdLuc and Adp53 viability curves that overlapped, while a greater than additive effect of Ad p53 and drug would result in an Adp53 curve that fell below the AdLuc viability curve. An example of a greater than additive effect is illustrated in Figure 1A below, where T47D cells were treated with Adp53 or Adßgal together with the DNA damaging drug, cisplatin.

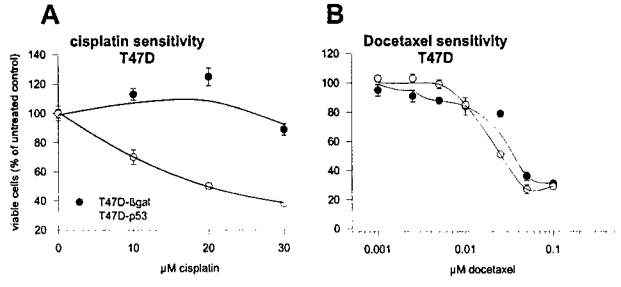


Figure 1. 96-well viability assay (see text) of T47D cells treated with Adp53 or Adß-gal (100 pfu/cell 3 hours) followed by treatment with (A) cisplatin or (B) taxotere (docetaxel) at the indicated doses for 1 hour or 3 hours, respectively. Cells were then incubated an additional 5 days and viability was scored by the MTS assay. Points represent the average of quadruplicate wells, and are expressed as a percentage of control wells (vector alone, no drug).

The greater than additive effect suggests a mechanism whereby p53 enhances the response of the tumor cells to DNA damage. An example of a simple additive effect is illustrated in Figure 1B, where T47D cells were treated with Adp53 and the microtubule inhibitor, taxotere (docetaxel). The simple additivity suggests p53 and taxotere kill cells through independent mechanisms that do not interfere. Table 3 summarizes the results of these and other assays. The results show that ectopic over expression of wild-type p53 in breast cancer cells following treatment with a p53 adenovirus, sensitizes these cells to a variety of DNA damaging treatments, irrespective of endogenous mutant p53 status. These results are consistent with earlier results with U.V. and ionizing radiation, which result in DNA crosslinks and double strand DNA breaks, respectively, and suggest that wild-type p53 is able to trigger growth arrest or apoptosis in tumor cells in response to several different types of DNA damage. In the wild-type p53-expressing cell line, MCF7, the effects observed were additive. Overall the results support he application of adenovirus-mediated gene transfer of p53 to a broad range of tumor types, and suggest that the suppressive effects of p53 would be additive to greater than additive with most chemotherapies.

Table 3
Results of viability assays testing the combination effects
of Adp53 and various chemotherapeutic drugs in breast cancer cell lines

	*Efficacy of drug combination with Adp53**			
Cell Line:	*Cisplatin*	*Taxotere*	*Doxorubicin*	*5-fluorouracil*
T47D	++	+	++	++
MDA MB 231	++	+	++	+
MCF7	+	+	+	+
MDA MB 435	++	n.d.	+	++

* ++ (greater than additive); + (additive); n.d. (not determined)

Treatment of cells with Adp53 results in abnormally high expression of p53 (see for example, Figure 9, below), a situation that may not always be achieved In order to determine how the therapy response of breast cancer cells would be affected by co-expression of wild-type and mutant p53 at roughly similar levels of expression, we selected subclones of T47D cells stably modified with wild-type p53 following transfection with plasmid vectors for constitutive transgene expression or with vectors for inducible transgene expression, as described in Progress Reports 1,2. The plasmid vector for constitutive expression was the pCEP4 vector (Invitrogen, Inc., Carlsbad CA), which does not integrate but maintains stable expression under selection (hygromycin) by virtue of an independent eucaryotic origin of replication (EBV origin). We prepared a pCEP4 vector encoding wild-type p53 (pCEPp53), transduced T47D cells, selected several hygromycin resistant clones and characterized them for wild-type p53 expression by Western analysis and by a CAT reporter assay. The reporter assay used the PG13 plasmid developed by Vogelstein and colleagues in which expression of chloramphenicol acetyltransferease is driven by an upstream wild-type p53-responsive element. While levels of total p53 expression were low, and although cell growth was not altered, expression of wild-type p53 could be demonstrated in one clone by the reporter assay. In 96 well growth assays, we found that the wild-type p53-modified clone was more sensitive to cisplatin and to 5-fluorouracil than was a control vector modified clone, but did not show enhanced sensitivity to taxotere (docetaxel), consistent with the viability assays with Adp53 (Table 3).

In order to facilitate the selection of wild-type p53-expressing clones and circumvent the growth disadvantage of high levels of wild-type p53 expression, we used an inducible two-vector system (Invitrogen, Inc.). In this system, the p53 transgene is cloned downstream of an ecdysone (muristerone)-inducible promoter, which is under the control of a transcription factor supplied by the second vector. The system offered the potential advantages of low background expression, highly inducible expression following treatment with muristerone, and suitability for both in vitro and in vivo studies. Despite these potential advantages, we did not find that the system facilitated the selection of clones stably modified with wild-type p53. We found that a pool of wild-type p53-expressing T47D clones, as well as one isolated clone, were able to induce wild-type p53 expression following treatment with 1 μM muristerone, as assayed by the PG13CAT assay. Levels of induced expression were similar to what had been observed in the T47D clone modified with pCEPp53. The pooled clones were found to have increased sensitivity to cisplatin following treatment with muristerone.

Although we used Adp53 to carry out most of the studies described here, the work with the stably modified cells expressing low levels of wild-type p53 demonstrated that the sensitization effect of wild-type p53 does not require high levels of expression. Thus, under conditions where wild-type p53 and mutant p53 are expressed at roughly similar levels, a condition that might exist transiently during tumor progression, or under treatment conditions where gene transfer by Adp53 was inefficient, the cell nevertheless displays a therapy-sensitive phenotype.

TECHNICAL OBJECTIVES II, III. MECHANISTIC STUDIES.

Task 2: Correlate DNA damage levels with sensitivity to p53-mediated effects, using DNA damage assays. The results of the viability assays carried out in task 1 show that p53 sensitizes tumor cells to DNA damaging chemotherapeutic agents and DNA damaging radiation, but not to

taxotere, whose primary target is the mitotic apparatus rather than DNA. This supports our hypothesis that the level of DNA damage is a key determinant of p53-mediated apoptosis.

To obtain further support for this hypothesis, we made use of a series of cells lines (derived from T98G glioblastoma cells) with a specific disruption in the Jun Kinase pathway that results in an altered DNA damage response, decreased DNA repair, increased genome instability, and increased levels of endogenous DNA damage (4, 5). The cell lines enabled us to explore how levels of endogenous DNA damage contribute to p53-mediated apoptosis, and how we might exploit inhibition of the Jun Kinase pathway in combination with p53 to achieve increased apoptosis. Consistent with our hypothesis, these cell lines with a defective Jun Kinase response pathway were also more sensitive to p53-mediated apoptosis. The results are presented in detail in a published manuscript (reference (4), attached), and are summarized here.

Disruption of the Jun Kinase pathway. To disrupt the Jun Kinase pathway, one of the key cellular DNA-damage response pathways, the cells were modified with a non-phosphorylatable mutant of the c-Jun transcription factor. c-Jun is a component if the heterodimeric AP-1 transcription complex (jun/fos) and the Jun/ATF2 transcription complex. Activation of these transcription complexes occurs when c-Jun undergoes N-terminal phosphorylation (on serines 63 and 73) by Jun Kinase in response to DNA damage (including damage caused by cisplatin). Although the molecular consequences of this activation are not fully understood, it is clear from our work that the pathway is critical in promoting survival following DNA damage, and is required for DNA repair.

The nonphosphorylatable c-Jun mutant has alanine substitutions at positions 63 and 73 and therefore fails to be modified in response to DNA damage. However, because the mutant can still enter into complexes with its heterodimeric partner, it competes with c-Jun and acts as a dominant-negative inhibitor of c-Jun downstream targets. T98G glioblastoma cells that express mutant Jun (verified by Western analysis of total Jun, see reference (4)) have an increased sensitivity to the DNA damaging drug cisplatin that correlates with an inability to repair DNA (5), suggesting that c-Jun downstream targets (including AP-1 regulated genes) might be involved in promoting DNA repair and survival following DNA damage. We have extended this observation to MDA MB 231 breast cancer cells modified with retroviral vectors as discussed in Progress Report #4. In addition, we have shown that MDA MB 231 breast cancer cells modified with a c-Jun antisense vector have a similar defect in repair of DNA damage. In all cases, expression of the transgene (c-Jun, m-Jun, c-Jun antisense) was confirmed by Western analysis of total Jun. Finally, we have shown that cells treated with dicoumarol, an inhibitor of Jun Kinase activation, also prevents repair of DNA damage. The results of the viability assays and DNA repair assays of T98G cells and MDA MB 231 cells are shown in Figure 2. Together, these results argue for an important role for the Jun Kinase pathway in the survival response to DNA damage via an effect on DNA repair, and provide evidence that the mutant Jun modification enhances p53-mediated apoptosis through an effect on DNA repair.

Analysis of DNA repair in cells with disruptions in the Jun Kinase pathway. We have analyzed DNA repair by two methods, a host cell reactivation assay and a PCR-stop assay, described in Progress Report #1. The PCR-stop assay has also been described in detail in our publications (references (4, 6), attached). The host cell reactivation assay measures the ability of transfected cells to restore activity of a reporter plasmid that has been damaged ex-vivo by treatment with cisplatin. A drawback of the assay is that it measures only repair of an exogenous fragment of plasmid DNA,

rather than nuclear repair of genomic DNA in chromatin. The PCR-stop assay has the advantage of measuring damage on a genomic region of DNA in chromatin, and may therefore more accurately reflect cellular DNA repair activity. Although we have found similar results with both assays when we have compared them, we have chosen to rely on the PCR-stop assay for the analyses shown in Figure 2. Briefly, the assay is based on the observation that platinum adducts block the progression of the Taq polymerase used for PCR amplification. By amplifying quantitatively a rather large fragment of DNA (in our case, a 2.7 Kb fragment of the HPRTase gene), we are able to detect a marked difference in the PCR signal strength between genomic DNAs prepared from control cells and cisplatin-treated cells. The recovery of the PCR signal as a function of time following cisplatin treatment provides a measure of DNA repair capacity. As shown in Figure 2, disruption of the Jun Kinase pathway following modification with mutant Jun, with c-Jun antisense, or with dicoumarol, leads to an inhibition of DNA repair that correlates with decreased survival following cisplatin treatment.

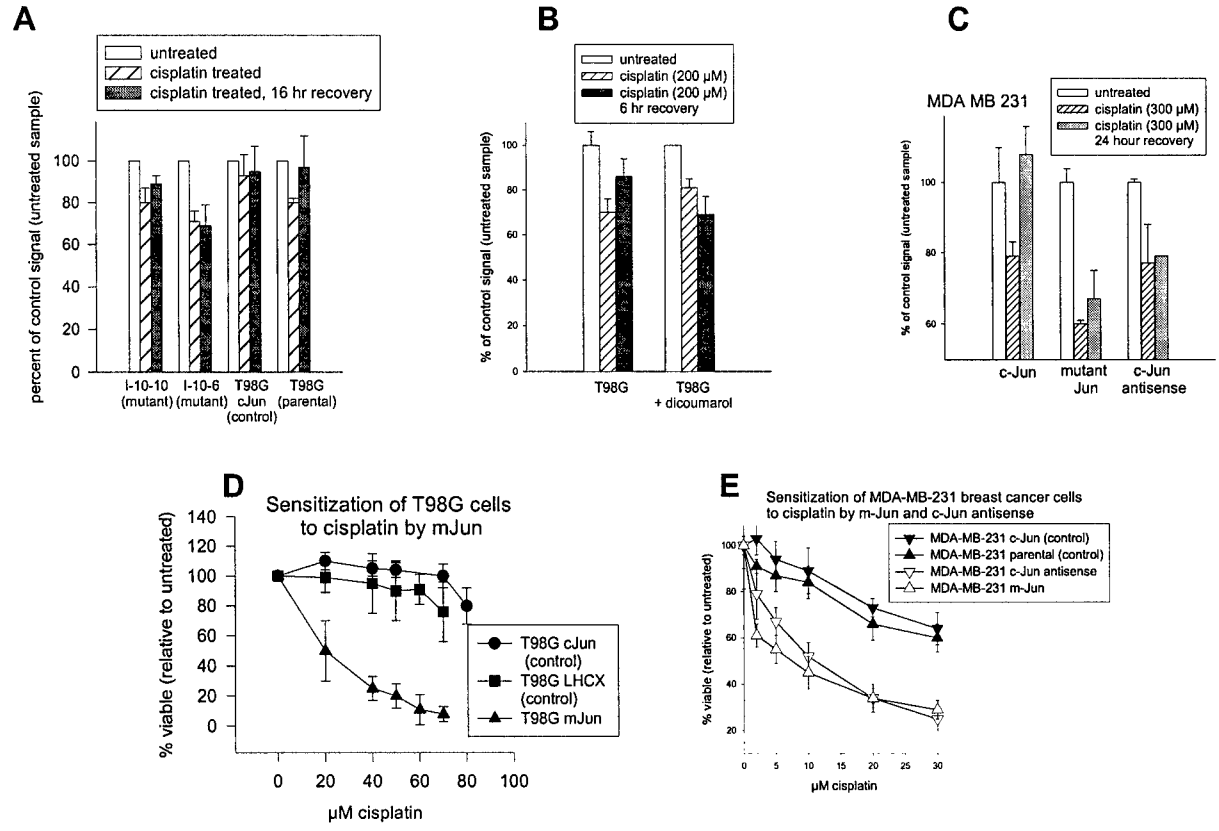


Figure 2. PCR-stop DNA repair assays (A-C) and viability assays (D,E) of T98G cells and MDA MB 231 cells following treatment with cisplatin. Cells were treated 1 hour with 100 µM, 200 µM, or 300 µM cisplatin, respectively in A, B, C. Cisplatin treatment conditions were chosen empirically to provide a sufficiently high level of damage to be detectable in the DNA damage assay. Bars represent the PCR signal strength relative to undamaged genomic DNA. Viability assays in D, E were carried out in 96 well plates as described for task 1, and represent viability remaining 5 days post treatment (1 hour treatment). Each point is the average of quadruplicate wells.

Evidence that mutant Jun-modified cells have elevated levels of endogenous DNA damage. Certain types of DNA repair defects contribute to increased genome instability (7, 8). We examined the T98G clones described above for DHFR gene amplification, one measure of genome instability. T98G cells were plated in the presence of concentrations of methotrexate 5 times the LD50 and 9 times the LD50 determined for these cells, i.e., concentrations at which gene amplification of

DHFR is known to be the predominant mechanism of resistance to the cytocidal effects of methotrexate (9, 10). Thus the frequencies of appearance of methotrexate-resistant clones is a measure of genome instability. As shown in Table 4, T98G mutant Jun clone I-10-10 produces methotrexate resistant colonies at about 20 times the frequency of the parental T98G cells, and T98G mutant Jun clone I-10-6 produces methotrexate resistant colonies at about 80 times the frequency of parental T98G cells (p=0.006). In contrast, stable expression of wild-type c-Jun had no effect on the frequency of resistance indicating strongly that interference with a phosphorylation-dependent function of c-Jun predisposed cells to form resistant colonies.

In order to verify the occurrence of gene amplification in methotrexate resistant colonies, several colonies were picked from each selection condition and expanded. Genomic DNA was prepared and subjected to quantitative PCR analysis using ^{32}P-labeled primers that define a 270 base fragment of the DHFR gene which includes part of exon 1 and intron A. PCR products were analyzed by agarose gel electrophoresis and quantitated by radioanalytic imaging as described in Material and Methods. The relative increase in PCR product from cellular DNA of methotrexate-resistant cells compared to unselected parental T98G cells was taken as a measure of the increased copy number of the DHFR gene and is indicated in Table 4 (last column). The methotrexate resistant clones derived from T98G mutant Jun expressing clones have from 2 to 4 times the gene dosage of the DHFR gene relative to parental T98G cells, indicating that the observed methotrexate resistance reflected an increased DHFR gene copy number. We conclude therefore, that inhibition of DNA repair by mutant Jun in T98G glioblastoma cells leads to accumulated DNA damage which can promote gene amplification.

Table 4.

Frequency of methotrexate(MTX)-resistant colonies in T98G cells and in cJun and dn-Jun-modified cells.

Cell were plated at a density of 10^5 cells per 10-cm culture dish and allowed to 4 weeks in the presence of methotrexate at the indicated concentrations. Plates were then stained with 70% methylene blue in methanol and colonies were counted. For analysis of DHFR gene amplification, several colonies were picked at random prior to staining, expanded and cellular DNA was prepared and subjected to PCR analysis

Clone	*MTX dosea*	*frequency of colonies per 10^5 cells (average of 3 experiments)*	*DHFR gene dosage relative to parental*
T98G parental	5 x LD50	2.0	1
	9 x LD50	0.3	
c-Jun	5 x LD50	2	n/t
	9 x LD50	0	
dnJun I-10-10	5 x LD50	40	3-4
	9 x LD50	10	
dnJun I-10-6	5 x LD50	174	2
	9 x LD50	16	

a) LD50 = 0.1 μM (T98G, c-Jun) or 0.04 μM (dnJun I-10-10, dnJun I10-6).

Mutant Jun-modified cells are more sensitive to p53-mediatedgrowth suppression and apoptosis. Based on the observations above indicating that mutant Jun-expressing cells are inhibited in DNA damage repair and predisposed to gene amplification, we predicted that strand breaks would accumulate in mutant Jun-expressing cells thereby leading to increased p53-dependent growth inhibition and apoptosis. Figure 3 compares the growth inhibition of Ad-p53-transduced cells relative to Adßgal-transduced cells 6 days post-infection. The results represent the average of two experiments performed on separate occasions, with each experiment being performed in triplicate. The infection efficiency, determined by X-gal staining of parallel cultures treated with Adßgal, was about 50% in all cases, low enough to cause incomplete growth suppression of parental T98G cells and control cells modified to stably express wild-type c-Jun as shown in Figure 3. Growth studies revealed that T98G mutant Jun clones I-10-10 and I-10-6 cells were considerably more growth

suppressed upon expression of p53 under these conditions. Western blot analysis (not shown) of the p53-responsive gene product, $p21^{waf1/cip1}$ in cell lysates 48 hours post-infection showed induction of $p21^{waf1/cip1}$ in all cases, indicating that p53 was expressed in those cells. The data thus show that $p21^{waf1/cip1}$ itself is not a crucial player in this setting.

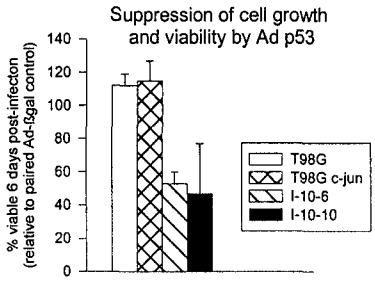


Figure 3. 6 day viability assay of T98G subclones following treatment with Ad-p53, 100 pfu/cell for 3 hours. Viability of Ad-p53-treated cultures is represented as a percentage of the same culture treated under identical conditions with Ad-ßgal.

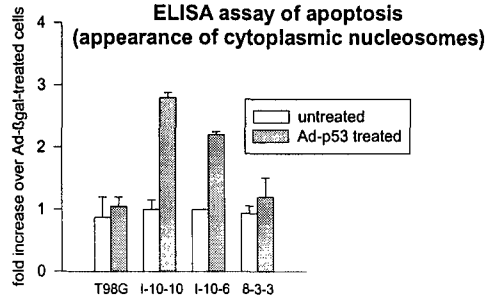


Figure 4. ELISA apoptosis assay of cytoplasmic nucleosomes in untreated cells, or in cells 48 hours after being treated with 100 pfu per cell of Ad-ßgal or Ad-p53 for 3 hours.

In order to determine whether the p53-mediated growth inhibition of T98G mutant Jun expressing cells shown in Figure 3 could be accounted for by the induction of apoptosis, we assayed the cytoplasmic fractions of Ad-p53 or Ad-ßgal-infected cells, 48 hours post-infection, for the presence of oligonucleosomal fragments (Figure 4). These fragments are released from the nuclei of cells undergoing apoptosis, and can be detected by an ELISA assay using anti-histone antibodies and anti-DNA peroxidase antibodies. We assayed for apoptosis 48 hours following exposure to p53-adenovirus or ß-gal adenovirus as this is the point at which we have observed maximal transgene expression in Ad-ß-gal-infected cells (unpublished observations). Figure 4 shows the results of the ELISA assay on the various T98G cell clones. Low levels of oligonucleosomal fragment release similar to levels observed in uninfected cells were observed in Ad-ß-gal-infected cells. Treatment of parental T98G cells and control wild-type c-Jun–expressing T98GcJun cells with Ad-p53 (100 pfu/cell, 3 hours) resulted in virtually no induction of apoptosis under our conditions, consistent with growth assays showing no suppression of overall growth following treatment of these cell lines with Ad-p53. However, readily detectable and significantly increased levels of apoptosis were observed in mutant Jun expressing clones I-10-10 and I-10-6. Thus p53-mediated apoptosis is markedly enhanced in mutant Jun-expressing cells, possibly as a consequence of being triggered by endogenous strand breaks that fail to be repaired.

To confirm a p53-dependent mechanism of apoptosis, we carried out a Western blot analysis of the apoptosis regulatory proteins bax and bcl_2 in cells treated with Ad-p53 or Ad-ßgal. The levels of the proapoptotic effector, bax, whose gene is induced by p53 (11), increase following treatment with Ad-p53, as expected, while levels of the antiapoptotic protein bcl_2 remain largely unchanged. A comparison of the bax to bcl_2 protein is indicated by the ratios under the lanes in Figure 5. The bax/bcl_2 ratio following treatment with Adp53 is significantly higher in mutant Jun-expressing cells I-10-10 and I-10-6 (ratios of 10 and 2.5, respectively) than in parental cells (ratio of 1.7) and c-Jun control cells (ratio of 0.8). Furthermore, a comparison of these ratios in uninduced *versus* induced

cells (Adßgal-treated *versus* Adp53-treated) reveals a 3 to 4-fold increase for the Adp53-treated parental and cJun-expressing control cells compared to the same cells treated with Adßgal, whereas Adp53-treated mutant Jun-expressing cells I-10-10 and I-10-6 show an increase of some 8 to 25 fold, respectively, compared to the same cells treated with Adßgal. In accordance with other data suggesting that the bax to bcl_2 ratio is a critical determinant of apoptosis (see (12), review), these data support a role for bax in the increased apoptosis observed after Adp53 treatment of mutant Jun-expressing cells.

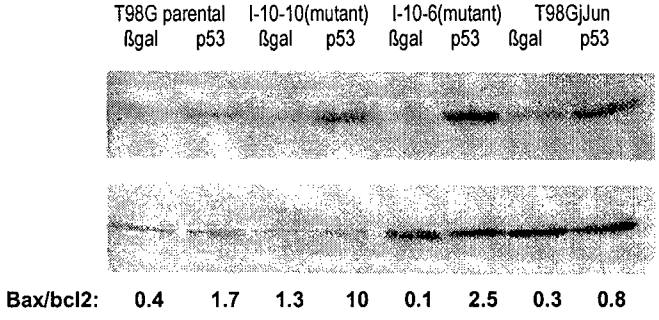


Figure 5. Western blot analysis of bax and bcl_2 protein in lysates from T98G parental cells, mutant Jun-expressing clones I-10-10 and I-10-6, and control c-Jun-expressing clone T98GcJun, 48 hours after treatment with Ad-ßgal or Adp53, 100 pfu per cell for 3 hours. Each lane represents 15 µg protein for the bax analysis and 30 µg protein for the bcl_2 analysis. Following immunostaining and band detection with ECL Western reagent, bands were quantitated using Kodak digital software. Ratios of bax to bcl_2 are indicated below the lanes. Experiment was carried out twice with similar results.

Model correlating DNA damage to disruptions in the Jun Kinase pathway and to p53-mediated apoptosis. These results have been published (reference (4)) and can be understood in light of a growing body of evidence supporting a role for p53 in modulating apoptosis in response to DNA damage (see review, ref. (13)), and in proportion to the extent of damage (14). p53 is a DNA damage recognition protein known to bind to a variety of types of DNA damage, including single stranded ends (15), and insertion-deletion loops (16). These types of damage, which could serve as triggers for p53-mediated apoptosis are likely to be generated in tumor cells by the mechanisms that promote spontaneous gene rearrangements, deletions, and amplifications. As such, a failure of DNA repair in mutant Jun-expressing cells would promote the accumulation of strand breaks, which would, on the one hand, favor gene amplification and other manifestations of genome instability, and on the other hand, promote DNA damage-induced stabilization of p53 and apoptosis. The scheme illustrating our original hypothesis can therefore be expanded as in Figure 6 (taken from reference (4)) to incorporate these results.

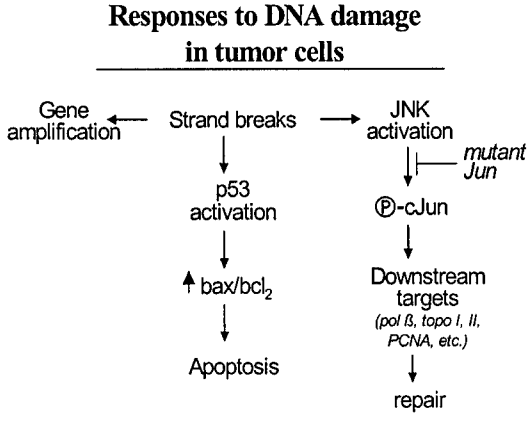


Figure 6. Model explaining how, through inhibition of potential c-Jun downstream targets leading to DNA repair (e.g., DNA polymerase ß, topoisomerase I, II, PCNA), mutant Jun promotes the accumulation of endogenous DNA strand breaks in genomically unstable tumor cells and thus collaborates with p53 to promote p53-mediated induction of bax and apoptosis.

Task 3: Correlate AP-1 activity and DNA repair activity with sensitivity to p53-mediated effects, using assays for AP-1 regulated genes associated with DNA repair. The correlation of DNA repair activity to sensitivity to p53-mediated growth suppression and apoptosis has been addressed in the DNA damage studies discussed under task 2. In task 3 we attempted to correlate changes in AP-1 regulated gene activity with the DNA repair defects accompanying disruptions in the Jun Kinase pathway. We have used RT-PCR to analyze how expression of a number of genes involved in DNA synthesis and/or repair was altered following disruption of the Jun Kinase pathway. The genes analyzed are listed in Table 5 below, and contain within their promoters or intronic sequences putative binding sites for AP-1 or ATF2/CREB transcription complexes (based on a TESS search for transcription factor recognition sequences). For RT-PCR, total cellular RNA was prepared from about 5 x 10^5 exponentially growing cells using the Rneasy™ kit from Qiagen and following the manufacturer's procedure. 1 μg of RNA was reverse transcribed into cDNA in a 20 μl reaction containing 0.5 mM dNTPs (Pharmacia), 100 μg/ml oligo dT (Promega), 2 units RNAsin (Promega), 10 units Moloney Murine leukemia virus reverse transcriptase (Promega), reverse transcriptase buffer (Promega). This cDNA was then used as a template for quantitative PCR. Primers have been chosen in all cases to amplify a region of about 200 bp in length from the coding sequence of the gene. Primers were synthesized by Genosys, Inc. Amplification conditions are as described above for the PCR-stop assay. Prior to carrying out an analysis of gene expression levels, pilot experiments were performed to verify quantitative conditions for each primer pair by preparing cDNA from 100 ng and 10 ng RNA (in addition to 1 μg RNA) and amplifying the cDNA to show that product formation was proportional to input template under our conditions. Following amplification, products were analyzed by agarose gel electrophoresis followed by band quantitation using a Kodak digital camera. For each of the genes shown in Table 5, expression (relative to actin message) either before or after treatment with cisplatin, was not significantly altered by expression of mutant Jun. Consistent with the RT-PCR results, we did not observe differences in the enzymatic activities of topoisomerase I, nor polymerase ß in parental or mutant Jun-modified cells (not shown).

Table 5
Genes whose expression (by RT-PCR) in T98G cells
was not altered by mutant Jun modification.

Gene	*Function*	*Expression change after cisplatin**
ERCC1	Damage recognition, Excision repair	n/c
GADD45	Interacts with PCNA, p53 regulated, required for DNA repair.	Increase
GADD153	Damage induced transcription factor	n/c
DNA pol ß	Bypass synthesis	Increase
Topoisomerase I	DNA cleavage religation-DNA synthesis and repair	n/c
PCNA	Proliferating cell nuclear antigen, DNA polymerase accessory protein	n/c

* Measured 18 hours after 1 hr treatment with 200 μM cisplatin. n/c = no change.

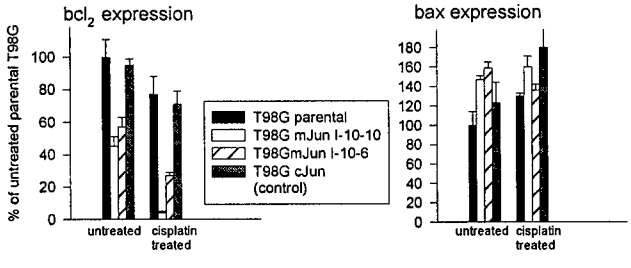


Figure 7. RT-PCR analysis of basal and cisplatin-induced expression levels of the bcl_2 and bax genes in T98G cells, T98G m-Jun I-10-10 and I-10-6, and T98G c-Jun cells (control). Bars represent expression relative to untreated T98G parental cells.

We have also examined expression of the p53 gene (mutant in these cells), as well as expression of the p21wafl and bax genes. Expression of p21wafl increased after treatment of cells with cisplatin, irrespective of mutant Jun status, indicating that induction of p21wafl can occur by p53-independent and by c-Jun independent mechanisms. However, overall basal and induced levels of p21 were higher in cells modified with mutant Jun. It has recently been reported that c-Jun knockout murine fibroblasts express elevated levels of p21 due to loss of c-Jun mediated transcriptional repression of the p53 gene (17). As shown in Figure 7,we observe differences in the regulation of the anti-apoptotic gene, bcl_2, suggesting that altered regulation of bcl_2 may contribute to the altered apoptotic response in mutant Jun modified cells. Basal levels of bcl_2 expression are significantly suppressed in mutant Jun modified cells relative to parental cells and c-Jun modified cells. Upon treatment with cisplatin, bcl_2 levels decrease even more, exacerbating the differences between clones that express mutant Jun and those that do not. Thus, while there is no evidence that bcl_2 is directly involved in DNA repair, it may contribute indirectly to DNA repair by delaying apoptosis. Its reduced expression in mutant Jun-modified cells is therefore consistent with our earlier observations of decreased repair in these cells and enhanced p53-mediated apoptosis.

These results begin to identify some of the common molecular events in two independently derived mutant Jun-modified T98G clones that may contribute to the phenotype we observe. The results provide further rationale for searching for other genes, possibly c-Jun downstream targets involved in DNA repair, whose deregulated expression would affect the way cancer cells respond to induced DNA damage and to p53-mediated apoptosis.

In order to clarify the possible role of AP-1 regulated genes in modulating DNA repair and p53-mediated apoptosis, we carried out experiments with the anti-AP-1 retinoids (18), 9-cis retinoic acid, and two synthetic retinoids, SR1120 and SR 11327, provided by Magnus Pfahl, Sidney Kimmel Cancer Center. The synthetic retinoids were found to have superior anti-AP-1 activity without the generalized toxicity associated with naturally occurring retinoids such as 9-cis retinoic acid (18). Retinoids are potential anti cancer agents and we have observed in breast cancer cells a greater than additive suppression by the synthetic retinoid SR1120 in combination with p53. We also observed an additive to greater than additive effect of SR1120 in combination with cisplatin. The similarities in the biological effects of retinoids in combination with p53 gene replacement or with cisplatin was similar to what we had observed with a variety of approaches to disrupting the Jun Kinase pathway, and we asked whether a common mechanism might be involved. Figure 8 plots the results of a combinaton experiment using Adp53 with 9-cis retinoic acid, where we observed a combined effect similar to the one we had observed with SR1120.

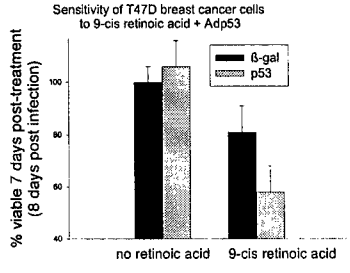


Figure 8. Viability of Adp53-treated or Adß-gal-treated T47D breast cancer cells in the presence or absence of 9 cis-retinoic acid. Cells were treated with vector at 50 pfu per cell for 3 hours, and replated in 96-well plates at 1000 cells per well. The next day, quadruplicate wells were either lefyt untreated or were treated with 1 µM 9-cis retinoic acid. Cells were then incubated an addition 7 days. Viability was measured by the MTS assay.

We carried out DNA repair assays on genomic DNA prepared from T47D cells treated with cisplatin in the presence or absence of SR11327 to determine whether retinoids might be targeting the DNA repair machinery in a manner similar to what we observed following disruptions in the Jun

Kinase pathway. We observed that DNA repair became evident by 12 hours post-treatment with cisplatin and was not affected by the presence of SR11327. Therefore, under conditions where retinoid-related general toxicity is minimized, we do not observe a significant effect on DNA repair. This suggests that retinoids target a different pathway than is targeted by c-jun alterations described above, and that the two approaches synergize with p53 gene transfer through different mechanisms.

In carrying out these studies, we discovered a novel property of retinoids that might contribute to their ability to enhance the effects of cisplatin, and possibly to synergize with p53. This work was described in detail in Progress Report 2 and in reference (6) (attached), and is summarized here. The experiments were based on published reports showing that cisplatin induces a structural distortion in DNA when it forms adducts (19). Because the initiation of transcription involves a structural distortion in DNA, we hypothesized that the transcription complex might present a preferential target for cisplatin-adduct formation. This would be consistent with several studies suggesting that transcription is a key target for cisplatin and that transcriptional repression may correlate better with cisplatin toxicity than does inhibition of DNA synthesis. We used the same PCR-based assay that we had used for DNA repair assays to examine cisplatin adduct formation on the promoter and downstream regions of the retinoic acid receptor ß gene in T47D breast cancer cells, where this gene undergoes retinoic acid-dependent activation. We also examined two constitutively expressed genes as controls (HPRT and DHFR). Cells were treated with 9-cis retinoic acid, and then with increasing concentrations of cisplatin, and cisplatin adduct formation was examined on genomic DNA. We observed preferential platination of the retinoic acid receptor ß gene promoter relative to downstream regions of the same gene, and DHFR gene. This preferential platination was not observed in the absence of transcriptional activation of the gene, suggesting that a structural modulation occurring upon promoter activation may enhance cisplatin binding. Levels of platination per nucleotide were about 3 times higher on the activated promoter than on HPRT gene. The results suggest that chromatin remodeling following promoter activation may create sites that are preferentially vulnerable to cisplatin adduct formation. Retinoids may synergize with cisplatin by creating new targets for cisplatin binding in vulnerable genomic regions. Furthermore, abnormal transcriptional transactivation might be recognized by p53 as a trigger for apoptosis, and if so might contribute to the synergistic suppression observed by p53 in combination with retinoids.

Task 4: Identify Intermediates in p53-mediated apoptosis (RNA, protein analyses). As shown by the Western analysis in Figure 9, treatment of cells with Adp53 leads to overexpression of p53 assayed at 72 hours post infection, as expected, and to a striking induction of the p53 downstream targets mdm2, bax, and p21wafl. This induction of protein corresponds to a similar induction of message for these genes (not shown). For the T98G cell line (Figure 9), treatment with Adp53 resulted in 17% of the cells undergoing apoptosis, as determined by a Trypan Blue exclusion assay.

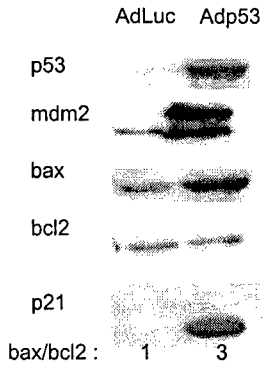


Figure 9. Western analysis of proteins induced following treatment of T98G cells for 3 hours with 500 pfu/cell of AdLuc (control) or Adp53. Lysates were prepared 72 hours post treatment. The bax to bcl2 ratio, which is known to increase in apoptotic cells, was determined by a digital analysis of the film.

In addition, as indicated in Figure 9, a digital analysis of the Western blot revealed a 3 fold increase in the bax to bcl2 ratio following Adp53 treatment. An increase in the bax to bcl2 ratio has previously been implicated as a feature of apoptotic cells. For us, the availability of mutant Jun modified subclones predisposed to p53-mediated apoptosis enabled us to correlate apoptosis with specific changes in gene expression. As discussed in Task 3, we found that changes in the bax to bcl2 ratio (Figure 5), but not changes in expression of p21, closely correlated with the increased apoptosis in the subclones. In some cell lines, a decrease in bcl2 expression following treatment with Adp53 contributed to the altered bax to bcl2 ratio. We observed similar changes in other cell lines, and conclude that the most relevant indicator of p53-mediated apoptosis is bax expression.

Task 5: Analyze the influence of the cell cycle on p53-mediated therapy sensitization (FACS analyses). In an effort to determine whether alterations in cell cycle distribution affected the response to p53, we carried out FACS analyses comparing cell lines with differing predispositions to p53-mediated apoptosis (T98G, T98G mutant Jun, T98G). We found that mutant Jun modified cell populations had a higher percentage of cells in the G2 phase of the cells cycle (Figure 10, top row, 67% in G2 versus 59%, respectively). Upon treatment with cisplatin, both cell populations accumulated in G2. However, parental unmodified cells returned to their control profile within 16 hours, while mutant Jun modified cells displayed a delayed recovery from G2 arrest. Abnormal regulation G2 arrest and recovery displayed by mutant jun modified cells may contribute the their increased predisposition to p53-mediated apoptosis.

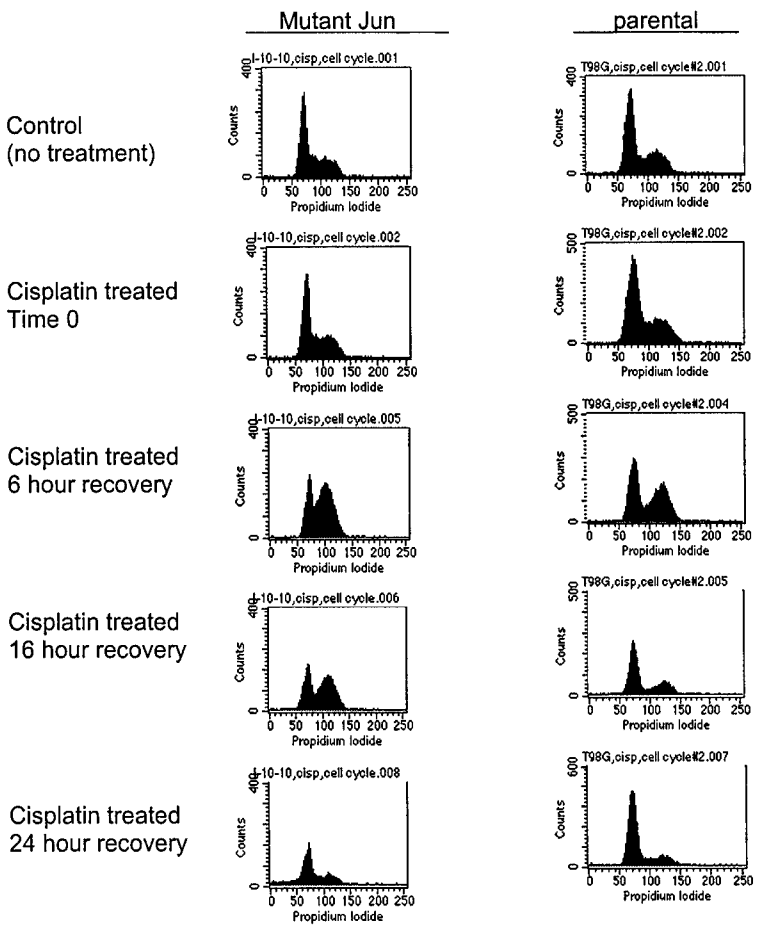


Figure 10. Cell cycle profile as analyzed by CellQuest software. Cells were treated with 30 μM cisplatin for 1 hour and then analyzed immediately (Time 0) or following a 6 hr, 16 hr, or 24 hr. recovery period.

TECHNICAL OBJECTIVE IV. *IN VIVO* STUDIES.

Task 6: Study the response of *ex vivo* transduced tumor cells to therapy using a subcutaneous tumor model. This study was not carried out, in light of concerns we had that such a model would not provide a rigorous evaluation of the clinical potential of p53-base therapies for breast cancer. Several published reports had demonstrated failure to achieve long-term transgene expression in vivo from cells modified ex vivo (20). To confirm these observations, we carried out a similar test of transgene expression in vivo, using the 9L rat glioblastoma cell line modified ex vivo to express wild-type p53 from the pCEP4 vector (the vector used in task 1). We found that while wild-type p53 expression in vitro could be maintained for up to one year or more (under selection), that in vivo expression was rapidly lost following orthotopic implantation of modified 9L cells (see PG13-CAT reporter assay for p53 expression, Figure 11). Another concern that prompted us to examine alternative models to the one originally proposed for this task, was that stable modification of cells with a tumor suppressor gene would select for low expression levels compatible with cell growth and survival. We did observe very low transgene expression by Western analyses of stably transduced clones studied in Task 1. Stably modified cell populations therefore seemed unlikely to express sufficiently high levels of transgene or to express the transgene for sufficiently long periods of time to provide a useful model for preclinical studies.

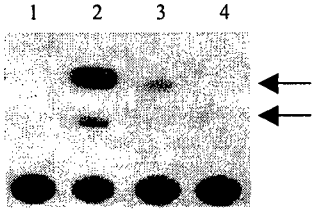


Figure 11. Thin layer chromatographic assay of CAT activity following transfection of various 9L cell subclones with the PG13 reporter plasmid for wild-type p53. In PG13, CAT expression is under the control of a p53-responsive element. The monoacetylated forms of chloramphenicol are indicated by the arrows. Lane 1: 9L cells; Lane 2: 9L cells transfected with a constitutive CAT reporter; Lane 3: 9L pCEPp53 cells grown in vitro; and Lane 4: 9L pCEPp53 cells after 3 weeks in vivo.

A model system more closely resembling a clinical treatment situation would involve a pre-established tumor into which a gene transfer vector was directly administered (Task 7). Since submitting the original grant proposal, an excellent model became available that offered not only the possibility of examining sub-cutaneous tumors, but also the possibility of examining orthotopic tumors with metastatic potential (MDA MB 435 metastatic model developed by Janet Price, University of Texas). The model therefore offers the opportunity to investigate therapeutic treatments for advanced disease, and is discussed below.

Task 7: Determine the response of established subcutaneous tumors to direct injection of wild-type p53 (± therapy). We examined the ability of a replication-defective adenovirus encoding wild-type p53 to suppress the growth of sub-cutaneous tumors of MDA MB 435 cells in female nude mice. Adenovirus was chosen for its unparalleled gene transfer efficiency and because of its promise as a clinical tool to achieve gene transfer in patients with cancer. Adenoviruses are not associated with serious pathogenicities, they infect a wide range of cell types, they can be obtained in high titers and they are stable, characteristics that together make adenoviruses attractive for clinical development. We obtained clinical grade adenoviruses encoding human wild-type p53 (Adp53) or firefly luciferase (AdLuc, control) from Introgen Therapeutics, Inc. (Houston, Texas).

Our in vitro studies (task 1, and Figure 12 A, below) had shown that greater suppression of tumor cell growth and viability in vitro was achieved by the combination of Adp53 plus the breast cancer

therapeutic, doxorubicin. Furthermore, apoptosis assays showed that the extent of apoptosis achieved by the combination of Adp53 and doxorubicin was greater than the sum of what was achieved by Adp53 or doxorubicin alone (see Figure 12 B).

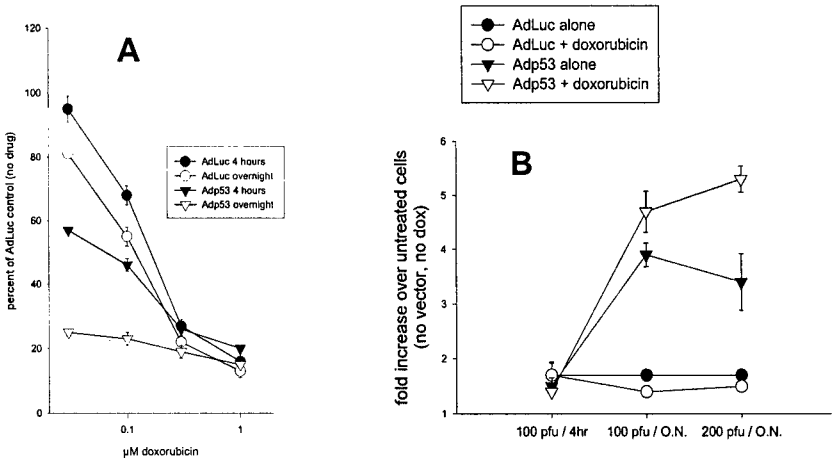


Figure 12 (A) Viability assay of MDA-MB-435 breast cancer cells treated as in Figure 1 with 100 pfu per cell of Adp53 or AdLuc for 4 hours or overnight, followed by replating in quadruplicate in 96 well plates. Following attachment, cells were treated with the indicated concentrations of doxorubicin (3 hours). Viability was assayed by the MTS assay 6 days from the start of infections. Viability is expressed as a percentage of the viability of cells treated only with AdLuc. (B) Apoptosis assay measuring release of oligonucleosomal fragments from the nucleus. Cells were treated as indicated with AdLuc or Adp53, with or without doxorubicin (0.1 µM) and cytoplasmic extracts were prepared 48 hours from the start of treatment and assayed for release of oligonucleosomal fragments. Results are expressed as the fold increase over untreated cells.

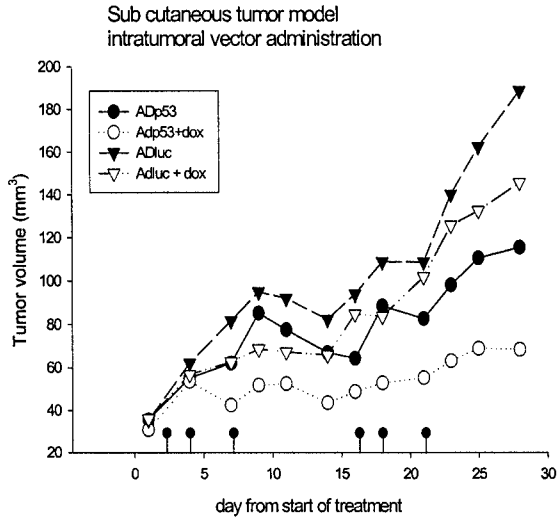


Figure 13. Subcutaneous model of MDA-MB-435 breast cancer in nude mice. Tumors were established in female nude mice by sub-cutaneous implantation of 10^6 cells and allowed to grow to an average size of 40 mm^3. Animals with established tumors were randomized into 4 treatment groups of 9 animals each and treatment was initiated (day 1). Each group received intratumoral injection of vector (3 x 10^8 pfu of AdLuc or Adp53 in 100 µl PBS) on days 2, 4, and 7 and again on days 16, 18, and 21 (vertical lines). Two groups also received intravenous injection of 100 µl of doxorubicin (8 mg/kg) on days 1 and 15 (lines). Tumor volumes were monitored at 2-3 day intervals.

We therefore included an experimental arm in the animal study in which tumors were treated with the Adp53 + doxorubicin combination. Tumors were initiated on the back of female nude mice (5-8 weeks) by subcutaneous implantation of 10^6 tumor cells in 100 µl DMEM. Tumor growth was monitored at 2-3 day intervals by measuring the length and width of the tumors and calculating tumor volume using the formula: volume = ½ length × $(width)^2$. When tumors had attained a size of 20-60 mm^3, animals were randomized into treatment groups of 9 animals each and treatment was initiated (designated day 1). Animals were administered 8 mg/kg doxorubicin (adriamycin, obtained through local pharmacies) on days 1 and 15 by tail vein injection. Adp53 or AdLuc (3 ×

10^8 pfu per tumor in 100 µl PBS) were administered on days 2, 4, 7, and again on days 16, 18, 21. Figure 13 plots the results of this experiment. The greatest suppression of tumor growth occurred in the Adp53 + doxorubicin treatment group over the entire period of the study. We found significant differences ($p < 0.05$ by a one-way analysis of variance followed by a pairwise Student t test) between the Adp53 group and the Adp53 + doxorubicin groups on days 7, 9, 11 (following the first cycle of treatment), and again on days 18, 23, 25, and 28 (following the second cycle of treatment). In addition, on days 18, 23, 25, 28 we also observed significant differences between the Adp53 + doxorubicin groups and each of the other treatment groups. No significant differences were observed on days 18 – 28 between the Adp53, AdLuc, and AdLuc + doxorubicin treatment groups. This indicates that Adp53, when administered directly to sub-cutaneous tumors is effective at reducing tumor growth and this effect is enhanced in the presence of doxorubicin.

The clone of MDA MB 435 cells used in this work was supplied to us by Janet Price (University of Texas, Houston) and had been selected by Price and coworkers for increased metastatic potential (primarily to lung) following orthotopic implantation into the mammary fat pads of female nude mice. Using this clone, we therefore had an opportunity to investigate the efficacy of Adp53 and doxorubicin in treating metastatic disease, a disease that poses the greatest challenge for conventional treatment.

Metastatic disease also poses a challenge for gene therapy approaches: systemic delivery of gene transfer vector would be required, but the potentially low gene transfer efficiency achievable following systemic administration could be a barrier to efficacy. Nevertheless, we had made several observations that suggested that therapeutic applications involving p53 might be particularly suited to overcoming the potential limitations of low gene transfer efficiency. First, we had observed (Task 1) that the tumor suppressive effects of p53 were enhanced in the presence of chemotherapeutic agents such as doxorubicin, and that combination effect was additive to synergistic. Furthermore, highly over-expressed wild-type p53 was not required to achieve therapy sensitization, as stably-modified clones of breast cancer cells, where wild-type p53 expression was too low to have a significant impact on growth, nevertheless displayed an increased sensitivity to chemotherapy. Finally, when a ß-galactosidase adenovirus was administered intravenously, and animals were sacrificed 2 days later and lungs were observed for X-gal staining (indicating transfer of the ß-galactosidase gene), we observed patches of stained areas. Taken together these results show that systemically delivered adenovirus will reach the lung and achieve gene transfer, and that the therapy sensitization effect of p53 observable even at low levels of expression could offset a potentially low gene transfer efficiency of systemic administration.

We therefore set up a metastatic model for human breast cancer in nude mice following a protocol developed by Janet Price. 10^5 MDA MB 435 cells were implanted under the skin of the mammary fat pad of female nude mice. Tumors were allowed to develop for 8 weeks, at which time most of the tumors were expected to have metastasized to the lung, based on prior work by Price and colleagues. Tumors that were large (> 800 mm^3) were excised (although residual tumor remained) and animals were randomized into treatment groups of 6 to 8 animals each. Treatment was then initiated (designated day 1). Animals received doxorubicin (10 mg/kg) by tail vein injection on days 1 and 21, and vector (Adp53 or AdLuc) beginning on day 3 and continuing at 2-3 day intervals until day 46. A total of 2×10^{10} viral particles were administered per injection. This corresponded to 3×10^8 pfu per injection for Adp53 and 10^9 pfu per injection in the case of AdLuc. At 15-16 weeks

post implantation the animals were sacrificed and the lungs were removed and fixed in formaldehyde. Longitudinal and transverse sections were prepared and analyzed for the presence of metastatic masses. As shown in Table 6, lung metastases were observed in all treatment groups except the group that had been treated with Adp53 + doxorubicin. Using the Fisher exact test to analyze the statistical significance of the differences observed, we found a significant difference in the incidence of metastases between the Adp53 group and the Adp53 + doxorubicin group ($p < 0.05$), as well as between the Adp53 group and the doxorubicin group ($p < 0.05$). This indicates that the Adp53 + doxorubicin combination could reduce the incidence of metastases. No significant differences were observed in the incidence of metastases between the no treatment and doxorubicin only groups, nor between the AdLuc and AdLuc + doxorubicin groups. (Table 6). These results are consistent with the in vitro results showing that the Adp53 + doxorubicin combination was more effective at promoting apoptosis than was either Adp53 or doxorubicin alone. Overall toxicity of the treatment was minimal, as assessed by weighing the animals at the end of treatment, and by histological examination of fixed liver sections. The results of the subcutaneous and orthotopic breast cancer tumor models have been published ((21)copy attached).

Table 6
Incidence of lung metastases in nude mice, 16 weeks following mammary fat pad implantation of 10^5 MDA-MB-435 breast cancer cells.
(% of animals with metastases)

Animal group	N/group	Incidence (histology) (%)	P value	Average animal weight (gm) per group
No treatment	8	38	P > 0.5	26
Dox	8	50		26
Adp53	8	50	P < 0.05	26
Adp53 + dox	7	0		24
AdLuc	8	38	P > 0.5	26
AdLuc + dox	6 (2 deaths)	17		26

Treatment was initiated at 8 weeks post-implantation of tumor cells, and just after excision of the primary tumors. Treatment consisted of vector, 10^{11} viral particles (10^9 pfu) of p53 adenovirus (or AdLuc control) administered 3 times per week by tail vein injection. Some of the animals received doxorubicin intravenously (100 µl of a 2 mg/ml stock, or 10 mg/kg) on days 1 and 21.

KEY RESEARCH ACCOMPLISHMENTS

- p53 gene transfer has broad application as a tumor suppressor and therapy sensitizer for breast cancers of differing endogenous p53 status.

- The suppressive effects of p53 and antimitotic agents are additive, while the suppressive effects of p53 and several agents known to damage DNA (including the standard breast cancer therapeutic, doxorubicin) are greater than additive, consistent with a DNA damage-dependent mechanism of action for p53.

- The therapy sensitization effect of wild-type p53 is manifested even at low levels of wild-type p53 expression, suggesting that therapeutic applications involving p53 might be particularly well suited to overcoming the potential limitations of low gene transfer efficiency in vivo.

- An important determinant of p53-mediated apoptosis is the level of endogenous DNA damage, and this is increased by treatment of cells with inhibitors of DNA repair. p53-mediated apoptosis correlates with and increase in the bax to bcl2 ratio, but not with increased expression of the downstream p53 target, p21wafl.

- Disruption of the Jun Kinase pathway inhibits DNA repair, increases the frequency of gene amplifications, and leads to increased apoptosis through the bax pathway following restoration of p53 activity.

- Treatment of breast cancer cells with retinoids leads to increased cisplatin adduct formation on the promoter region of the retinoic acid receptor ß gene, following activation by 9-cis retinoic acid. This suggests that retinoids such as 9-cis retinoic acid, through their pleiotropic effects on gene expression, generate new targets for cisplatin, and enhance its cytotoxicity through inhibition of the transcription initiation complex. Abnormal transcriptional transactivation by retinoids may also promote p53-mediated apoptosis.

- Intratumoral administration of a replication impaired adenovirus encoding wild-type p53 suppresses the growth of established subcutaneous tumors of human breast cancer cells in nude mice, and this suppression is enhanced in the presence of the standard breast cancer therapeutic, doxorubicin (adriamycin).

- Systemic administration of a replication impaired adenovirus encoding wild-type p53 in combination with doxorubicin results in a significant reduction in the incidence of lung metastases, using an orthotopic model in nude mice for human metastatic breast cancer.

REPORTABLE OUTCOMES

U.S. Patents

"Down-Regulation of DNA Repair to Enhance Sensitivity to p53-Mediated Apoptosis." (Ruth. A. Gjerset, inventor) (issued April 25, 2000)

Abstracts/posters (supported in whole or in part by the DOD)

1. "Tumor Suppressor Therapy for Metastatic Breast Cancer" (poster) Huang, Y., Saadatmandi, N., Haghighi, A., Gjerset, R.A.
   (to be presented) California Breast Cancer Research Symposium, Sacramento, CA, September 2001,

2. "p53-based therapies for breast cancer" (abstract) Gjerset, R.A., Haghighi, A., Lebedeva, S., Bagdasarova, S., Tyler, T.
   Era of Hope Meeting Sponsored by the DOD, Atlanta, Georgia, June 8-12, 2000.

3. "T98G glioblastoma cells defective in DNA repair show enhanced p53-mediated growth suppression and apoptosis". (poster) Haghighi, A., Lebedeva, S., Mercola, D., Gjerset, R.A.
   American Association for Cancer Research Special Conference on DNA Repair Defects, San Diego CA, January 14-18, 2000.

4. "The cellular stress response modulates sensitivity to p53." (poster and invited oral presentation) Gjerset R.A., Mercola, D., Lebedeva, S., and Turla, S.T.
   Gordon Research Conference on Cancer, August 2-7, 1998, Newport, RI.

5. "Preferential DNA binding of cis-diamminedichloroplatinum to an activated promoter." (poster) Haghighi, A., Lebedeva, S., Gjerset, R.A.
   Conference on chromatin structure and function, June 16, 1998, Paris, France.

6. "Therapy Sensitization of Breast Cancer by p53" (poster and invited Platform presentation). Gjerset, R.A., Haghighi, A., Lebedeva, S., Turla, S.T.
   Proceeding of the DOD Era of Hope Meeting 3: 817.
   Era of Hope Meeting Sponsored by the DOD, Oct 31-Nov 4, 1997, Washington DC.

7. "Suppression of glioblastoma by p53 combination therapy." (poster) Gjerset, R.A., Dorigo, O., Turla, S.T., Lebedeva, S.
   Proceedings of the eighty-eighth annual meeting American Association for Cancer research 1997, 38: 431.
   88th Annual meeting of the AACR, April 12-16th 1997, San Diego CA.

8. "The JNK/SAPK pathway regulates DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin." (poster) Potapova,O., Gjerset, R.A., Haghighi, A., Bost, F.,Turla, S.T., Birrer, M, Mercola, D.
   Proceedings of the eighty-eighth annual meeting American Association for Cancer research 1997, 38: 139., 88th Annual meeting of the AACR, April 12-16th 1997, San Diego CA.

9. "Tumor Regression in vivo following p53 combination therapy". (poster and invited General session presentation), Gjerset, R.A. Dorigo, O., Maung, V., Lebedeva, S. Turla, S.T.
Cancer Gene Therapy 1997 4(6): pp O-2
Sixth International Conference on Gene Therapy of Cancer, Nov 20-22, 1997, Coronado CA

10. "Inhibition of the JNK/SAPK pathway is a novel approach for sensitizing human tumor cells to chemo- and radiotherapy and for inhibition of tumor growth". (poster) Potapova, O., Bost, F., Haghighi, A.,Gjerset, R.A., Dean, N., Mercola, D.
Cancer Gene Therapy pp O-12.Sixth International Conference on Gene Therapy of Cancer, Nov 20-22, 1997, Coronado CA

11. "Inhibition of jun N-terminal kinase pathway sensitizes human tumor cells to chemotherapy and radiotherapy" (poster) Potapova, O., Bost, F., Gjerset, R.A., Turla, S.T., Haghighi, A., Birrer, M., Mercola, D.
9th Lorne Cancer Conference (Special joint Conference with the AACR), February 13-16, 1997, Lorne, Victoria, Australia

12. "The Jun kinase pathway functions to regulate DNA repair and growth, and inhibition of the pathway by antisense oligonucleotides sensitizes tumor cells to cisplatin," (poster) Potapova, O., Haghighi, A., Bost, F., McCay,R., Dean, N., Gjerset, R.A., Mercola, D.
12th Annual San Diego Conference of the American Association of Clinical Chemists, November 1997, San Diego, CA

13. "p53-suppression potentiated by inhibition of AP-1 phosphorylation," (poster and oral presentation) Turla, S.T., Potapova, O., Lebedeva,S., Haghighi, A., Bost, F., Mercola, D., Gjerset, R.A.
Cancer Gene Therapy 1996, 3(6) pp P77
Fifth International Conference on Gene Therapy of Cancer, Nov 14 -16, 1996, Coronado, CA.

14. "Enhancement of anti-tumor effects of p53 gene therapy by combination with DNA-damaging agents" Harper, M.E., Christiano, R, Spitz, F., Nguyen, D., Gjerset, R.A., Roth, J.A.
Cancer Gene Therapy 1996 3(6) pp P114.
Fifth International Conference on Gene Therapy of Cancer, Nov 14 -16, 1996, Coronado, CA.

15. "Effects of wild-type p53 expression in cells inhibited in DNA repair" (poster) Gjerset, R.A., Turla, S.T., Potapova, O., Mercola, D.
Eighth International p53 workshop. Dundee, Scotland July 5th - 9th, 1996.

Manuscripts

1. Gjerset, R.A. and Sobol, R.E. (1997) Treatment resistance, apoptosis and p53 tumor suppressor gene therapy. In: Encyclopidia of Cancer, J.R. Bertino, ed., Academic Press, San Diego, vol. III,

pp. 1785-1791.

2. Potapova, O., Haghighi, A., Bost, F., Liu, C., Birrer, M.J., Gjerset, R., Mercola, D. (1997) The jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. J. Biol. Chem., 272:14041-14044.

3. Haghighi, A., Lebedeva, S., Gjerset, R.A. (1999) Preferential platination of an activated cellular promoter by cis-diamminedichloroplatinum. Biochemistry 38: 12432-12438.

4. Gjerset, R.A., Lebedeva, S., Haghighi, A., Turla, S.T., Mercola, D. (1999) Inhibition of the Jun Kinase pathway blocks DNA repair, enhances p53-mediated apoptosis and promotes gene amplification. Cell Growth and Differ. 10: 545-554.

5. Gjerset, R.A. and Mercola, D. (2000) Sensitization of tumors to chemotherapy through gene therapy. *In* "Cancer Gene Therapy: Past Achievements and Future Challenges", N. Habib, editor, Plenum Publishing Corp., New York, London, Moscow. Pp. 273-291.

6. Lebedeva, S., Bagdasarova, S., Tyler, T., Mu, X., Wilson, D.R., and Gjerset, R.A. (2001) Tumor suppression and therapy sensitization of localized and metastatic breast cancer by adenovirus p53. Human Gene Therapy 12: 763-772.

7. Gjerset, R., Haghighi, A., Lebedeva, S., Mercola, D. (2001) Gene therapy approaches to sensitization of human prostate cancer to cisplatin. *In* "Methods in Molecular Biology: Genomics Protocols", M. Starkey and E. Elaswarapu, eds. The Humana Press, Inc., Totowa, N.J. (*in press).*

8. Saadatmandi, N., Wilson, D.R., Gjerset, R.A. (2001) Therapeutic Applications of a p53 Gene Therapy vector, Ad5CMV-p53. *In* Encyclopedia of Cancer, 2nd edition, J. Bertino, ed., Academic Press, San Diego. (*in press)*

Funding applied for based on work/experience supported by the DOD award

1. A Novel Tumor Suppressor Approach to Cancer Therapy" (principal investigator) California Cancer Research Program 1/1/99 – 12/31/00 (funded)

2. "Role of p14ARF in Metastatic Breast Cancer"(principal investigator Ruth Gjerset, Co-investigator, Per Borgstrom) California Breast Cancer Research Program 7/01/00 – 6/30/02, (funded)

3. "Triple Combination Therapy for Breast Cancer Metastases" (principal investigator, Ruth A. Gjerset) U.S. Army Medical Research Command Breast Cancer Research Program.7/1/01 – 6/30/4 (not funded)

4. "Novel activities of p14ARF in cancer" (principal investigator, Ruth A. Gjerset) National Institutes of Health) 4/1/02-3/31/07 (pending)

CONCLUSIONS

We have shown that restoration of wild-type p53 activity sensitizes breast cancer cells to a variety of DNA damaging treatments, including doxorubicin, a standard breast cancer therapeutic. These observations extend our earlier observation on p53-mediated sensitization to cisplatin, and suggest a broad applicability of p53 as a general sensitizer to DNA damaging therapies.

Using clonal derivatives of the T98G cell line, and the MDA MB 231 breast cancer cell line, modified so as to be suppressed in N-terminal c-Jun phosphorylation, and defective in DNA repair, we have demonstrated a correlation between decreased DNA repair, increased genome instability, and increased sensitivity to p53-mediated apoptosis through the bax pathway. This result supports our central hypothesis that DNA damage constitutes a key determinant of a tumor cell's susceptibility to p53-mediated apoptosis, and provides additional rationale for the combined use of p53 with DNA repair inhibitors, as well as DNA damaging drugs.

We have addressed the mechanism of synergy between retinoids and cisplatin, as therapeutic synergy between these two agents has been observed in tumor models for breast cancer, and because we have observed the restoration of p53 further improves the therapeutic efficacy of retinoids with cisplatin. Earlier published studies show that retinoid pretreatment enhances the total platinum binding to DNA of target cells. Using the RARß gene as a model, we have observed that the enhanced cisplatin binding occurs preferentially at the promoter region, suggesting that retinoids synergize with cisplatin by generating hypersensitive sites in promoter regions to which cisplatin preferentially binds, and possibly leading in this way, to the inhibition of transcription observed with cisplatin, followed by apoptosis. Because p53 is present in the transcription complex, it may be particularly sensitive to DNA abnormalities in these regions.

Using human breast cancer models in nude mice for local and metastatic disease, we demonstrated that administration of a replication impaired adenovirus encoding wild-type p53 was effective in combination with doxorubicin in suppressing primary tumor growth (following direct intratumoral delivery of vector) and suppressing metastatic spread of tumors to the lung (following systemic delivery of vector). We observed minimal toxicity of the adenoviral vector in animals over the course of the treatment period.

These studies have led to 8 scientific publications, 4 applications for further funding, and 15 presentations at scientific meetings as abstracts, posters, or oral presentations by the Principal Investigator. The work also contributed to one patent application filed for p53 combination therapies involving DNA repair inhibitors (issued April 2000).

REFERENCES

1. Scanlon, K. J., Kashani-Sabet, M., Tone, T., and Funato, T. Cisplatin resistance in human cancers, Pharmacol Ther. *52:* 385-406, 1991.
2. Scanlon, K. J., Jiao, L., Funato, T., Wang, W., Tone, T., Rossi, J. J., and Kashani-Sabet, M. Ribozyme-mediated cleavage of c-fos mRNA reduces gene expression of DNA synthesis enzymes and metallothionein, Proc Natl Acad Sci U S A. *88:* 10591-5, 1991.
3. Brachmann, R. K., Vidal, M., and Boeke, J. D. Dominant-negative p53 mutations selected in yeast hit cancer hot spots, Proc Natl Acad Sci U S A. *93:* 4091-5., 1996.
4. Gjerset, R. A., Lebedeva, S., Haghighi, A., Turla, S. T., and Mercola, D. Inhibition of the Jun kinase pathway blocks DNA repair, enhances p53- mediated apoptosis and promotes gene amplification, Cell Growth Differ. *10:* 545-54, 1999.
5. Potapova, O., Haghighi, A., Bost, F., Liu, C., Birrer, M. J., Gjerset, R., and Mercola, D. The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin, J Biol Chem. *272:* 14041-4, 1997.
6. Haghighi, A., Lebedeva, S., and Gjerset, R. A. Preferential platination of an activated cellular promoter by cis- diamminedichloroplatinum, Biochemistry. *38:* 12432-8, 1999.
7. Lengauer, C., Kinzler, K. W., and Vogelstein, B. Genetic instability in colorectal cancers, Nature. *386:* 623-7, 1997.
8. Melton, D. W., Ketchen, A. M., Nunez, F., Bonatti-Abbondandolo, S., Abbondandolo, A., Squires, S., and Johnson, R. T. Cells from ERCC1-deficient mice show increased genome instability and a reduced frequency of S-phase-dependent illegitimate chromosome exchange but a normal frequency of homologous recombination, J Cell Sci. *111:* 395-404., 1998.
9. Brown, P. C., Tlsty, T. D., and Schimke, R. T. Enhancement of methotrexate resistance and dihydrofolate reductase gene amplification by treatment of mouse 3T6 cells with hydroxyurea, Mol Cell Biol. *3:* 1097-107., 1983.
10. Tlsty, T. D., Brown, P. C., and Schimke, R. T. UV radiation facilitates methotrexate resistance and amplification of the dihydrofolate reductase gene in cultured 3T6 mouse cells, Mol Cell Biol. *4:* 1050-6., 1984.
11. Miyashita, T. and Reed, J. C. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene, Cell. *80:* 293-9., 1995.
12. Basu, A. and Haldar, S. The relationship between BcI2, Bax and p53: consequences for cell cycle progression and cell death, Mol Hum Reprod. *4:* 1099-109., 1998.
13. Harris, C. C. Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies, J Natl Cancer Inst. *88:* 1442-55., 1996.
14. Chen, X., Ko, L. J., Jayaraman, L., and Prives, C. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells, Genes Dev. *10:* 2438-51., 1996.
15. Bakalkin, G., Selivanova, G., Yakovleva, T., Kiseleva, E., Kashuba, E., Magnusson, K. P., Szekely, L., Klein, G., Terenius, L., and Wiman, K. G. p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain, Nucleic Acids Res. *23:* 362-9, 1995.

16. Lee, S., Elenbaas, B., Levine, A., and Griffith, J. p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches, Cell. *81:* 1013-20, 1995.
17. Schreiber, M., Kolbus, A., Piu, F., Szabowski, A., Mohle-Steinlein, U., Tian, J., Karin, M., Angel, P., and Wagner, E. F. Control of cell cycle progression by c-Jun is p53 dependent, Genes Dev. *13:* 607-19., 1999.
18. Fanjul, A., Dawson, M. I., Hobbs, P. D., Jong, L., Cameron, J. F., Harlev, E., Graupner, G., Lu, X. P., and Pfahl, M. A new class of retinoids with selective inhibition of AP-1 inhibits proliferation, Nature. *372:* 107-11., 1994.
19. Takahara, P. M., Rosenzweig, A. C., Frederick, C. A., and Lippard, S. J. Crystal structure of double-stranded DNA containing the major adduct of the anticancer drug cisplatin, Nature. *377:* 649-52., 1995.
20. Scharfmann, R., Axelrod, J. H., and Verma, I. M. Long-term in vivo expression of retrovirus-mediated gene transfer in mouse fibroblast implants, Proc Natl Acad Sci U S A. *88:* 4626-30., 1991.
21. Lebedeva, S., Bagdasarova, S., Tyler, T., Mu, X., Wilson, D. R., and Gjerset, R. A. Tumor suppression and therapy sensitization of localized and metastatic breast cancer by adenovirus p53, Hum Gene Ther. *12:* 763-72., 2001.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 22, Issue of May 30, pp. 14041–14044, 1997
Printed in U.S.A.

Communication

The Jun Kinase/Stress-activated Protein Kinase Pathway Functions to Regulate DNA Repair and Inhibition of the Pathway Sensitizes Tumor Cells to Cisplatin*

(Received for publication, January 6, 1997, and in revised form, April 1, 1997)

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We have studied the role of Jun/stress-activated protein kinase (JNK/SAPK) pathway in DNA repair and cisplatin resistance in T98G glioblastoma cells. JUN/SAPK is activated by DNA damage and phosphorylates serines 63 and 73 in the N-terminal domain of c-Jun, which is known to increase its transactivation properties. We show that treatment of T98G glioblastoma cells with cisplatin but not the transplatin isomer activates JNK/SAPK about 10-fold. T98G cells, which are highly resistent to cisplatin (IC_{50} = 140 ± 13 μM), modified to express a nonphosphorylatable dominant negative c-Jun (termed dnJun) exhibit decreased viability following treatment with cisplatin, but not transplatin, in proportion ($r_{Pearson}$ = 0.98) to the level of dnJun expressed leading to a 7-fold decreased IC_{50}. Similar effects are observed in U87 cells, PC-3 cells, and MCF-7 cells, as well as in T98G cells modified to express TAM-67, a known inhibitor of c-Jun function. In contrast, no sensitization effect was observed in cells modified to express wild-type c-Jun. Furthermore, through quantitative polymerase chain reaction-stop assays, we show that dnJun expressing cells were inhibited in repair of cisplatin adducts (p = 0.55), whereas repair is readily detectable (p = 0.003) in parental cells. These observations indicate that the JNK/SAPK pathway is activated by cisplatin-induced DNA damage and that this response is required for DNA repair and viability following cisplatin treatment. Regulation of DNA repair following genotoxic stress may be a normal physiological role of the JNK/SAPK pathway.

* This work was supported by Grant CA 63783 from the National Cancer Institute (to D. A. M.), by La Ligue Nationale Contre le Cancer (to F. B. and D. A. M.), by the Tobacco-Related Diseases Research Program of California (to R. G.), by the U. S. Army Breast Cancer Research Project (to R. G.), by Introgen Therapeutics, Inc. (to R. G.), and by the Fellowship Program of the Sidney Kimmel Cancer Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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JNK/SAPK[1] is part of a kinase cascade that phosphorylates the transcription factor c-Jun at serine residues 63 and 73 (1–9). Phosphorylation of c-Jun at these sites greatly enhances the transactivation potential of the AP-1 binding sites (1–4) and AP-1 regulated genes *in vivo* (5, 11, 12), and there is evidence suggesting roles for c-Jun phosphorylation in cellular transformation (1, 2), inflammation (14), and apoptosis (15). The JNK/SAPK pathway is strongly stimulated in a dose-dependent manner by various DNA damaging treatments, including UV-C (5, 7–8), ionizing radiation (16), and alkylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (5), methylmethanesulfonate (MMS) (11), 1-β-D-arabinofuranosylcytosine (Ara-C) (17), and hydrogen peroxide (18). These observations suggest that the JNK/SAPK pathway may mediate a physiological response to DNA damage such as induction of one or more DNA repair enzymes. Here we provide evidence that the chemotherapeutic agent cisplatin, which damages DNA through the formation of bifunctional platinum adducts, but not transplatin, which does not damage DNA (19, 20), activates JNK/SAPK up to 10-fold in a dose-dependent manner. Furthermore, inhibition of this pathway in cells modified by expression of a nonphosphorylatable dominant negative mutant of c-Jun, dnJun, blocks DNA repair as judged by quantitative PCR and markedly decreases viability following treatment with cisplatin but not transplatin. Thus, JNK/SAPK is activated by cisplatin-induced DNA damage and is required for DNA repair and survival following cisplatin treatment.

EXPERIMENTAL PROCEDURES

Cells—Culture conditions and all cell lines and plasmids used here were developed using standard methods as described previously (22, 23). The expression of c-Jun and dnJun was quantitated using the methods (24) and antibodies previously characterized (24).

PCR—The PCR-stop assay (28) was used to quantitate cisplatin-DNA adduct formation and subsequent repair. The assay is based on the observation that the efficiency of amplification of cisplatin-treated DNA is inversely proportional to the degree of platination. Genomic DNA was isolated immediately or 6 h after treatment of cells for 1 h 15 min with varying amounts of cisplatin and amplified quantitatively using ^{32}P-end-labeled primers, giving rise to a 2.7-kb and a nested 0.15-kb fragment of the hyopxanthine phosphoribosyl transferase gene. The 5′ and 3′ primers were TGGGATTACACGTGTGAACCAACC and GATCCACAGTCTGCCTGAGTCACT, respectively, with a 5′ nested primer of CCTAGAAAGCACATGGAGAGCTAG. The 0.15-kb segment of genomic DNA sustains undetectable levels of DNA damage under our conditions and serves as an internal PCR control and the basis for normalization of the amount of amplification of the 2.7-kb fragment. The number of lesions/2.7-kb fragment (*i.e.* Fig. 4) is calculated as 1 − (cpm damaged DNA/cpm undamaged DNA) (8).

JNK Assay—JNK assays were carried out exactly as described previously (7).

Cytotoxicity—Viability (29) was assessed by the addition of cisplatin or transplatin for 1 h one day after seeding test cells into 96-well plates followed by a change of medium to fresh medium and determination of surviving cells 5 days later by addition of MTS for 1 h and determination of $A_{590\ nm}$ of the dissolved formazan product as described by the

[1] The abbreviations used are: JNK, c-Jun N-terminal kinase; AP-1, activator protein complex 1; cisplatin, *cis*-diaminodichloroplatinum; SAPK, stress-activated protein kinase; MTT, micro-tetrazolium (dye) test; transplatin, *trans*-diaminodichloroplatinum; UV-C, ultraviolet light C band, 254 nm maximum intensity for UV cross-linker 1800; dnJUN, dominant negative c-Jun; PCR, polymerase chain reaction; kb, kilobase pair; ATF, activation transcription factor; CREB, cAMP response element binding protein; MTS, (3-(4,5′-dimethylthiazol-2-yl)-5-(3-carboxymethoxylphenyl-2-(4-sulfophenyl)-2H-tetrazolium inner salt.

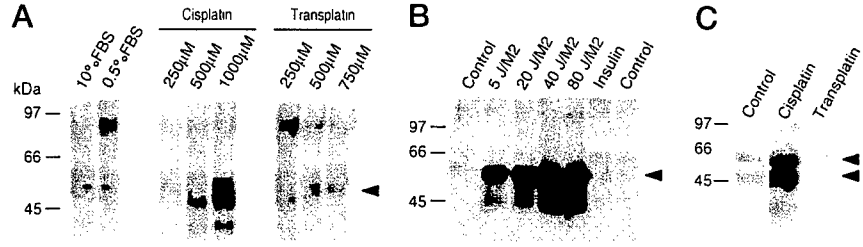

FIG. 1. **Cisplatin is a stereo-specific activator of JNK.** *A*, T98G human glioblastoma cells were stored overnight in serum-free medium, plated the next day, and treated by the indicated concentrations of cisplatin or transplatin for 1 h with a 1-h chase period followed by lysis and assay for JNK activity as described (7). Matching wells of cells were harvested and counted (Coulter counter) and used as the basis for sample loading. *FBS*, fetal bovine serum. *B*, positive control. T98G cells were exposed to the indicated doses of UV-C band (Stratalinker® UV cross-linker 1800) radiation and processed as described for *A*. *C*, JNK activity of human lung carcinoma M103 cells following treatment with 200 μM cisplatin or transplatin and processing as described for *A* with the addition of a 1-h chase prior to lysis.

manufacturer (Promega). All results were carried out in quadruplicate, and viability is expressed as the ratio of the amount of viable cells following cisplatin or transplatin treatment to that of the same cells without treatment.

RESULTS

Activation of JNK/SAPK by Cisplatin Requires DNA Adduct Formation—It is known that cisplatin but not transplatin forms covalent covalent cross-links between the N^7 position of adjacent guanine or adjacent adenine-guanine residues (19, 20). We find that the JNK activity of T98G cells is elevated in a dose-dependent manner up to 10-fold following a 1-h exposure to cisplatin but not to transplatin (Fig. 1*A*). As a positive control of the effects of a DNA-damaging agent, we examined the response of JNK of T98G cells to UV-C irradiation (Fig. 1*B*) and observed a similar dose-response relationship. Cisplatin-specific responses have been observed in other cell lines from tumor types that are commonly refractory to cisplatin treatment such as the human nonsmall cell lung carcinoma lines A549 (data not shown) and M103 (Fig. 1*C*). Moreover, 1 h after treatment with cisplatin, but not transplatin, JNK activity of T98G cells or lung carcinoma cells M103 remains elevated, suggesting that treatment with cisplatin leads to a prolonged response. These results indicate that only the DNA-damaging cisplatin isomer activates JNK activity.

Dominant Negative c-Jun Sensitizes Tumor Cells to Cisplatin but Not Transplatin—We developed clonal lines of human T98G glioblastoma cells, which stably express a dominant negative inhibitor (1, 2) of the JNK/SAPK pathway, dnJun. Expression of dnJun has no effect on either basal AP-1 activity (1, 2) or on the enzyme activity of JNK (data not shown) but does inhibit phosphorylation-dependent activation of transcription (1, 2, 10, 12). The effect of cisplatin treatment on the viability of representative clonal lines of the dnJun-expressing T98G cells is compared with that of an empty vector control line, T98GLHCX, in Fig. 2*A*. The viability of empty vector control T98G cells remains largely unaffected by treatment with increasing concentrations of cisplatin even at doses of ≥70 μM. Extended titrations revealed IC_{50} values of 147 and 154 μM for the parental cells and empty vector control cells, respectively (Table I). In contrast, the dnJun expressing cells exhibit an IC_{50} as low as 21 μM (Fig. 2*A*) or over 7-fold more sensitive to cisplatin than the control cells (Table I). Replicate experiments using additional clones that exhibit varying amounts of steady state dnJun indicate the sensitization to cisplatin is proportional ($r_{Pearson}$ = 0.98) to the amount of dnJun expressed (Fig. 2*B*). Transplatin has no discernible effect at concentrations where the viability following treatment with cisplatin is less than 25% (Fig. 3*B*) and in extended titrations no significant effect at 250 μM, indicating that the requirements for sensitization by dnJun depends upon the stereospecific DNA-binding properties of cisplatin, similar to the conditions for the activation of JNK.

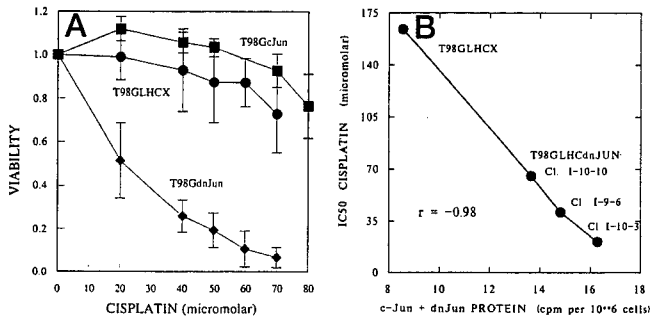

FIG. 2. **dnJun sensitizes T98G cells to cisplatin.** *A*, viability assay of empty vector control cells (●), wild-type c-Jun expressing cells (■), and clonal dnJun-expressing cells (♦). *B*, dose-response curve of the $IC_{50}^{cisplatin}$ of dnJun-expressing clones of T98G cells *versus* total immunoreactive c-Jun (*c-Jun* + *dnJun*). Total immunoreactive Jun (*c-Jun* + *dnJun*) was determined by sequential immunoprecipitation of ^{35}S-labeled cells using specific Jun B, Jun D, followed by pan-Jun antiserum as described previously (24). Values above 8.5 are taken as expression of dnJun.

Expression of wild-type c-Jun does not mimic dnJun-expressing cells (Fig. 2*A*). In fact, the viability of these cells when treated with cisplatin is somewhat *increased* relative to parental or empty vector control cells for all viability determinations in the range 20–60 μM cisplatin, suggesting that increased JNK substrate augmented viability following treatment with cisplatin (Fig. 2*A*). Thus, the sensitization to cisplatin observed for the dnJun-expressing cells appears to correlate with interference in the role of activated c-Jun.

Generality—We have tested the generality of the sensitizing properties of dnJun in PC3 prostate carcinoma cells modified to express dnJun under the control of an inducible truncated metallothionein promoter as described previously (21). Viability studies show that parental or empty vector control cells are largely insensitive to cisplatin at concentrations ≤60 μM (Fig. 3*A*, *circles*). Extended titrations revealed IC_{50} values of 109 and 156 μM for the parental and empty vector control cells, respectively (Table I). However, for PC3 cells that stably express pMTdnJun, the IC_{50} value is markedly reduced (Fig. 3*A*, *open diamonds*). Further, induction of maximum expression of dnJun by the addition of zinc acetate leads to greatly increased cytotoxicity with an IC_{50} of 16 μM (Fig. 3*B*) or 7.24–9.8-fold more sensitive to cisplatin than control cells (Table I). The addition of zinc acetate alone has no effect on the viability of parental or empty vector control cells (Fig. 3*A*, *filled circles*). Thus, the results observed following induction of expression of dnJun by a single clonal line confirm the results of Fig. 2*B* that sensitization to cisplatin is dependent upon the expression of dnJun.

As a further control, we examined PC3 prostate carcinoma cells modified to express a zinc-inducible c-Jun derivative, TAM-67, a well characterized transdominant negative inhibi-

TABLE I

Sensitization of human tumor lines to cisplatin-induced cytotoxicity

IC_{50} values were determined by direct titration of viability with cisplatin as described ("Experimental Procedures"). None of the cell lines examined here were made cisplatin-resistant prior to analysis.

Cell	Control[a]		dnJun-expressing IC_{50}	Cisplatin sensitization[b] $(IC_{50})^{Parent}/(IC_{50})^{dnJun}$
		IC_{50}		
		μM	μM	
T98G glioblastoma	Parental	140 ± 13	21 ± 3	7.0
	Empty vector pLHCX	154 ± 13		7.60
U87 glioblastoma	Parental	130 ± 53	50 ± 5	2.6
	Empty vector pLHCX	ND		
PC3 prostate carcinoma	Parental	109 ± 13	16 ± 2	7.2
	Empty vector pMT64AA	156 ± 18		9.2
MCF-7 breast carcinoma	Parental	145 ± 25	38 ± 2	3.8
	Empty vector pLHCX	101 ± 9		2.7

[a] In all cases parental and empty vector cells were analyzed in parallel and with equal concentrations of cisplatin and transplatin in the range 0–250 μM all in quadruplicate. Transplatin had no effect on viability of any cell. ND, not done.

[b] Sensitization is defined by the ratio of IC_{50} values for the parental or empty vector control cells to the IC_{50} value of the dnJun-expressing cells.

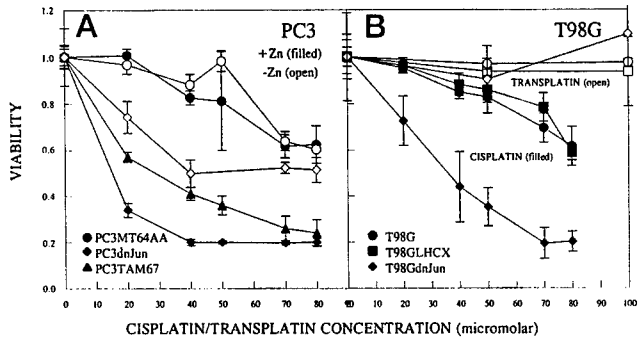

FIG. 3. **Sensitization of cells to cisplatin is general among cell types and stereospecific.** *A*, comparison of the viability of PC3 human prostate carcinoma cells modified to express pLHCX and the empty vector pMT64AA in the presence (■) or absence (●) of 25 mM zinc acetate to clonal PC3 cells the containing pLHCX and either the inducible vector pMTdnJun (◆) or pMTTAM-67 (▲) both in the presence of 25 mM zinc acetate. *B*, the viability of parental and modified T98G cells in the presence of cisplatin (*solid symbols*) or transplatin (*unfilled symbols*).

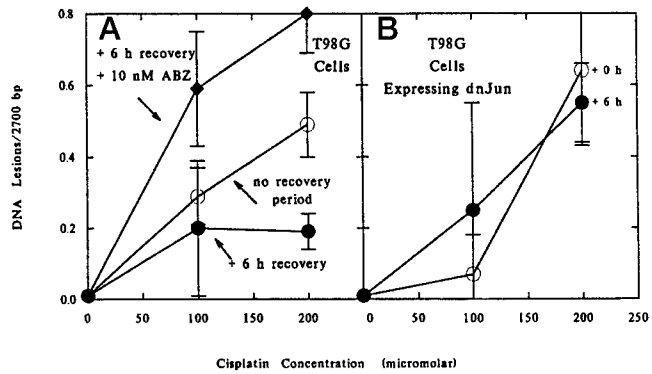

FIG. 4. **Expression of the transdominant inhibitor, dnJun, blocks cisplatin-induced DNA repair.** PCR results for the 2.7-kb segment of the hyopxanthine phosphoribosyl transferase gene was determined for 0, 100, or 200 μM cisplatin for 1 h as described ("Experimental Procedures") and expressed as 1 − (normalized efficiency of PCR amplification), a measure of cisplatin-induced lesions (10). *A*, PCR results for T98G parental cells either immediately or 6 h after treatment with cisplatin. The results are the averages of three assays for each of two independent preparations of DNA for the three concentrations of cisplatin. *B*, comparison of T98G cells and dnJun-expressing cells 6 h after treatment with cisplatin. The results are the averages of three assays. *ABZ*, 2-aminobenzidine.

tor of AP-1 owing to a deletion of residues 2–122 (21). As with dnJun, induction of TAM-67 in PC3 cells strongly enhances their sensitivity to cisplatin (Fig. 3*A*). We have determined that these TAM-67 and dnJun are expressed in approximately equal amounts, suggesting that the comparable degree of sensitization for TAM-67 and dnJun (Fig. 3*A*) is accounted for by interference in the role of phosphorylation-related function of c-Jun.

Similar results have been observed with an additional human glioblastoma line, U87, and an additional epithelial tumor line, MCF-7 (Table I). Clonal dnJun-expressing lines of these cells exhibit 2.6- and 3.8-fold decreased IC_{50} values, respectively (Table I). Thus, the sum of results indicate that the JNK/SAPK pathway may have a general role in mediating a functional response to DNA-cisplatin adduct formation. Inhibition of this response sensitizes cells to the cell-killing properties of cisplatin.

Cisplatin Activates and dnJun Inhibits DNA Repair—We assessed the extent of genomic DNA damage and repair following cisplatin treatment using a modified PCR assay (25). For this assay, it has been shown that the degree of inhibition of PCR-catalyzed amplification of DNA purified from *cisplatin-treated* cells is a direct measure of the amount of DNA-cisplatin adduct formation as measured by atomic absorption (25). Thus, this assay provides a direct assessment of the extent of cisplatin-induced DNA damage.

DNA isolated from T98G cells immediately after treatment with 0, 100, or 200 μM cisplatin for 1 h exhibit increasing levels of DNA damage (Fig. 4*A*, *circles*). However, if a 6-h "recovery" period is introduced prior to the DNA purification, damage is markedly and significantly ($p = 0.003$) reduced (Fig. 4*A*, *filled circles*). As a positive control for the effects of inhibition of genomic DNA repair, an inhibitor of ADP-ribosylation, 2-aminobenzidine, was added at the time of treatment of the cells with cisplatin (Fig. 4*A*, *squares*). Following the 6-h recovery period, DNA damage remained unrepaired, and total DNA damage was substantially increased. Next, we compared the level of DNA damage for T98G cells and dnJun-expressing cells following treatment with cisplatin (Fig. 4*B*). For the dnJun-expressing T98G cells, *6 h after* cisplatin treatment DNA damage remains completely unrepaired for cells treated at either 100 or 200 μM cisplatin ($p > 0.53$). All the results summarized here (Fig. 4, *A* and *B*) are the averages of three independent assays, which confirms the reliability of this observation. The sum of results, therefore, strongly indicates that expression of dnJun by T98G cells largely abolishes DNA repair following exposure of the cells to cisplatin.

DISCUSSION

These studies show that the JNK/SAPK pathway is activated by cisplatin-induced DNA damage and is required for DNA repair and viability following cisplatin treatment. T98G glioblastoma cells modified to express a nonphosphorylatable dom-

inant negative inhibitor of c-Jun, dnJun, fail to repair cisplatin adducts and are sensitized to the cytotoxic effects of cisplatin under conditions that have little or no effect on parental and control lines. In contrast, cell lines modified to overexpress wild-type c-Jun are resistant to cisplatin, an observation that rules out that possibility that the sensitization effect of dnJun is mediated by one or more of the domains it shares with wild-type c-Jun. Moreover, sensitization to cisplatin by dnJun is exhibited by several cell lines of varying origins. Sensitization to cisplatin is also observed in PC-3 prostate carcinoma cells modified to express TAM-67, a known dominant negative inhibitor of AP-1 (21). Because the degree of protein expression and sensitization is similar for TAM-67 and for dnJun, we conclude that most of the sensitization effects we observe are accounted for by inhibition of the phosphorylation-related functions of Jun.

Two major types of DNA regulatory elements that respond to the phosphorylation state of c-Jun include classic AP-1 sites and ATF/CREB sites. Classic AP-1 sites consisting of a 7-base pair consensus motif, T(G/T)A(C/G)TCA, bind to AP-1 complexes consisting of heterodimers of members of the Fos and Jun families and to Jun-Jun homodimers (9, 13–15, 21, 22, 26). ATF/CREB sites consisting of an 8-base pair consensus motif, T(G/A)CGTCA, bind to c-Jun/ATF2 heterodimers. Indeed, because JNK phosphorylates ATF2 as well as c-Jun and promotes complex formation and binding to ATF/CREB sites, these sites are likely to be major targets of JNK-mediated regulation (9, 12, 14, 15). Several enzymes known to be involved in repair of DNA-cisplatin adducts and implicated in cisplatin resistance (20) contain ATF/CREB sites in their promoters including DNA polymerase β (27, 28), topoisomerase I (30, 31), and proliferating cell nuclear antigen, an accessory protein of DNA polymerase delta (32, 33). Moreover, transcription of these genes is known to be activated through the ATF/CREB sites upon stimulation by genotoxic agents (27–33). Thus, the inhibition of induction of any or all of these activities could account for the inhibitory effects of dnJun on DNA repair and the resultant increase in cisplatin sensitivity. In view of the common regulatory mechanism involving ATF/CREB sites, a concerted induction of genes with a related function, DNA repair, is suggested. The sum of results indicate, therefore, that a potential physiological role for the strong activation of the JNK/SAPK pathway following DNA damage may be to mediate DNA repair by enhancing transaction of DNA repair enzymes.

Acknowledgments—We thank M. Karin for providing the glutathione *S*-transferase-c-Jun and dnJun expression vectors and Eileen Adamson for critically reading this manuscript and for support.

Addendum—During the review of this manuscript we became aware that activation of JNK/SAPK by cisplatin has been reported by Liu *et al.* (Liu, Z.-G., Baskaran, R., Lea-Chou, E. T., Wood, L. D., Chen, Y., Karin, M., and Wang J. Y. J. (1996) *Nature* **384,** 273–276).

REFERENCES

1. Smeal, T., Binétruy, B., Mercola, D. A., Birrer, M., and Karin, M. (1991) *Nature* **354,** 494–496
2. Smeal, T., Binétruy, B., Mercola, D. A., Bardwick-Grover, A., Heidecker, G., Rapp, U., and Karin, M. (1992) *Mol. Cell. Biol.* **12,** 3507–3513
3. Binétruy, B., Smeal, T., and Karin, M. (1991) *Nature* **351,** 122–127
4. Pulverer, B., Kyriakis, J. M., Avruch, J. Nikolakakie, E., and Woodgett, J., (1991) *Nature* **353,** 670–674
5. Dérijard, B., Hibi. M., Wu I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R., J. (1994) *Cell* **76,** 1025–1037
6. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) *Nature* **369,** 156–160
7. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes & Dev.* **7,** 2135–2148
8. Adler, V., Fuchs, S., Y. Kim, J., Kraft, A., King, M., P. Pelling, J., and Ronai, Z. (1995) *Cell Growth & Differ.* **6,** 1437–1446
9. Sluss, H., Barrett, T., Dérijard, B., and Davis, R. J. (1994) *Mol. Cell. Biol.* **14,** 8376–8384
10. van Dam, H., Wilhelm, D., Herr, I., Steffen, A., Herrlich, P., and Angel, P. (1995) *EMBO J.* **14,** 1798–1811
11. Adler, V., Franklin, C., and Kraft, A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5341–5345
12. van Dam, H., Duyndam, M., Rottier, R., Bosch, A., de Vries-Smits, L., Herrlich, P. Zantema, A., Angel, P., and van der Eb, A. J. (1993) *EMBO J.* **12,** 479–87
13. Whitmarsh, A., Shore, P., Sharrocks, A., and Davis, R. J. (1995) *Science* **269,** 403–407
14. Davis, R. (1994) *Trends Biochem. Sci.* **19,** 470–473
15. Xia, Z., Dickens, M., Raingeaud, J., Davis. R., and Greenberg, M. (1995) *Science* **270,** 1326–1331
16. Macgregor, P. F., Abate, C., and Curran, T. (1990) *Oncogene* **5,** 451–458
17. Kharbanda, S., Pandey, P., Ren, R., Mayer, B., Zon, L., and Kufe, D. (1995) *J. Biol. Chem.* **270,** 30278–30281
18. Yu, R., Jiao, J., Duh, J., Tan, T. H., and Kong, A. (1996) *Cancer Res.* **56,** 2954–2959
19. Sherman, S. E., Gibson, D., Wang, A. H., and Lippard, S. J. (1985) *Science* **230,** 412–417
20. Zamble, D. B., and Lippard, S. J. (1995) *Trends Biochem. Sci.* **20,** 435–439
21. Brown, P. H., Chen, T. K., and Birrer, M. J. (1994) *Oncogene* **9,** 791–799
22. Mercola, D. A., Carpenter, P., Grover-Bardwick, A., and Mercola, M. (1992) *Oncogene* **7,** 1793–1803
23. Potapova, O., H. Fakhrai, S. Baird and Mercola, D. (1996) *Cancer Res.* **56,** 280–286
24. Grover-Bardwick, A., Adamson, E., and Mercola, D. (1994) *Carcinogenesis* **15,** 1667–1674
25. Jennerwein, M. M., and Eastman, A. (1991) *Nucleic Acids Res.* **19,** 6209–6214
26. Karin, M., and Smeal (1992) *Trends Biochem. Sci.* **17,** 418–422
27. Kedar, P. S. Widen, S. G., Englander, E. W., Fornace, A. J. Jr., and Wilson, S. H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3729–3733
28. Horton, J., Srivastava, D. Zmudzka, B., and Wilson, S. H. (1995) *Nucleic Acids Res.* **23,** 3810–3815
29. Gjerset, R., Turla, S., Sobol, R., Scalise, J., Mercola, D., Collins, H., and Isabella, P. (1995) *Mol. Carcinogen.* **14,** 275–285
30. Heiland, S,. Knippers, R., and Kunze, N. (1993) *Eur. J. Biochem.* **217,** 813–822
31. Baumgartner, B., Heiland, S., Kunze, N., Richter. A., and Knippers, R. (1994) *Biochim. Biophys. Acta* **1218,** 123–127
32. Feuerstein, N., Huang, D., and Prystowsky, M. B. (1995) *J. Biol. Chem.* **270,** 9454–9458
33. Huang, D., Shipman-Appasamy, P. M., Orten, D., J., Hinrichs, S. H., and Prystowsky, M. B. (1994) *Mol. Cell. Biol.* **14,** 4233–4243

HUMAN GENE THERAPY 12:763–772 (May 1, 2001)
Mary Ann Liebert, Inc.

Tumor Suppression and Therapy Sensitization of Localized and Metastatic Breast Cancer by Adenovirus p53

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ABSTRACT

We have examined the effects of a replication-defective adenovirus encoding p53 (RPR/INGN 201 [Ad5CMV-*p53*]; Adp53), alone or in combination with the breast cancer therapeutic doxorubicin (Adriamycin), to suppress growth and induce apoptosis in breast cancer cells *in vitro*. We have also examined the *in vivo* effect of intratumoral administration of Adp53, alone or in combination with doxorubicin, to suppress the growth of established subcutaneous MDA-MB-435 breast cancer tumors. Finally, using the MDA-MB-435 orthotopic model of metastatic breast cancer, we have examined the effect of systemic administration of Adp53, alone or in combination with doxorubicin, to reduce the incidence of metastases. We find that whereas *in vitro* treatment of cells with Adp53 reduces [^{3}H]thymidine incorporation by about 90% at 48 hr, cell viability at 6 days is reduced by only some 50% relative to controls. Although apoptosis is detectable in Adp53-treated cultures, these results suggest that a large fraction of Adp53-treated cells merely undergo reversible cell cycle arrest. Combined treatment with Adp53 and doxorubicin results in a greater than additive loss of viability *in vitro* and increased apoptosis. *In vivo*, locally administered Adp53 suppresses growth of established subcutaneous tumors in nude mice and suppression is enhanced by doxorubicin. In the metastatic breast cancer model, systemic administration of Adp53 plus doxorubicin leads to a significant reduction in the incidence of metastases relative to Adp53 or doxorubicin alone. Taken together, these data indicate an additive to synergistic effect of Adp53 and doxorubicin for the treatment of primary and metastatic breast cancer.

OVERVIEW SUMMARY

Adp53 suppresses viability of MDA-MB-435 breast cancer cells *in vitro* by a mechanism that involves reversible cell cycle arrest in a fraction of cells and apoptosis in a fraction of cells. Apoptosis *in vitro* is enhanced by doxorubicin, and *in vitro* suppression of cell viability by the Adp53 plus doxorubicin combination is greater than additive. In established subcutaneous MDA-MB-435 breast cancer tumors in nude mice, doxorubicin enhances suppression by intratumorally delivered Adp53. In the MDA-MB-435 orthotopic model for metastatic breast cancer, doxorubicin plus Adp53 leads to a significant reduction in metastases compared with Adp53 alone or doxorubicin alone.

INTRODUCTION

ONE OF THE CHALLENGES for breast cancer treatment today is metastatic disease, which affects some 50% of breast cancer patients and is still essentially incurable, despite advances in diagnosis, staging, and management (Hendersin *et al.*, 1998). Metastatic disease not only evades conventional chemotherapy through acquired drug resistance, but it also poses a challenge for tumor suppressor gene therapy. Systemic delivery of gene transfer vectors would be required to treat metastatic disease, but the potentially low gene transfer efficiency achievable through systemic administration could be a barrier to efficacy.

A biological approach that may overcome the limitations of low gene transfer efficiency and have broad application to a large number of cancer patients, is p53 tumor suppressor gene therapy. Loss of p53 appears to play a central role in cancer, as it is one of the most frequent events in cancer (Nigro *et al.*, 1989). In breast cancer, loss or mutation of p53 occurs in some 25–40% of breast cancer patients (Bartek *et al.*, 1990; Coles *et al.*, 1992; Singh *et al.*, 1993) and has been associated with poor prognosis and shortened survival (Pharoah *et al.*, 1999; Cuny *et al.*, 2000; Powell *et al.*, 2000). In addition, loss of p53 correlates with increased resistance to DNA-damaging treatments

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in fibroblasts and hematopoietic cells from p53-null mice (Lotem and Sachs, 1993; Lowe *et al.*, 1993) and in tumors from patients (O'Connor *et al.*, 1997), consistent with the established role of p53 as a key mediator of apoptosis in response to stress and DNA damage (Eastman and Rigas, 1999; Lakin and Jackson, 1999; May and May, 1999). Numerous studies with a variety of tumor lines in culture and numerous animal tumor models *in vivo*, have confirmed that p53 gene transfer into tumor cells is not only suppressive in itself, but also enhances the antitumor effects of chemotherapy (Gjerset *et al.*, 1995; Dorigo *et al.*, 1998; Ferreira *et al.*, 1999; Lane, 1999; Gjerset and Mercola, 2000). Of particular interest is our observation that restoration of wild-type p53 expression in tumor cells that express endogenous mutant p53 can sensitize these cells to chemotherapeutic drugs, even when p53 is expressed at levels too low to significantly suppress growth by itself (Gjerset *et al.*, 1995; Dorigo *et al.*, 1998).

Because therapy sensitization occurring at low levels of p53 expression could offset the potentially low efficiency of systemic gene transfer, we reasoned that the therapy sensitization properties of p53 could be exploited even in the case of metastatic disease where systemic delivery would be necessary. p53 single-agent gene therapy for the treatment of several cancers has now entered the clinical trial phase (Clayman *et al.*, 1998; Roth *et al.*, 1999; Swisher *et al.*, 1999). Many approaches have employed adenovirus as a gene transfer vector, chosen for its unparalleled gene transfer efficiency and relative lack of toxicity (Seth *et al.*, 1999). However, most studies to date have examined the effects of local or regional vector administration to tumors, and have been largely limited to cancers that could benefit from such an approach; these studies, therefore, have not addressed the broader clinical problem of disseminated metastatic disease.

In the present study we have tested the efficiency of p53-mediated suppression of breast cancer and p53-mediated sensitization to the standard breast cancer therapeutic, doxorubicin, using both a subcutaneous tumor model involving intratumoral administration of Adp53 (RPR/INGN 201; Ad5CMV-*p53*), an adenoviral vector carrying the p53 gene, as well as a metastatic tumor model involving systemic administration of Adp53. We find that intramural administration of Adp53 to localized tumors results in suppression of tumor growth that is enhanced in the presence of doxorubicin. When Adp53 is delivered systemically in combination with doxorubicin to animals bearing mammary fat pad tumors, we observe a significant reduction in lung metastases. This indicates that potentially low levels of p53 expression following systemic administration need not be a barrier to effective application of p53 combination approaches for metastatic breast cancer.

MATERIALS AND METHODS

Cell line

A subclone of the MDA-MB-435 human beast cancer cell line selected for metastatic potential by passage through nude mice (Price, 1996) was provided by J. Price (M.D. Anderson Cancer Center, Houston, TX). MDA-MB 435 cells are estrogen receptor negative and express only mutant p53 with a Gly-to-Glu mutation at codon 266 (Lesson-Wood *et al.*, 1995).

Adenoviral vectors

The vectors AdLuc (control), Ad-βgal, and Adp53 (RPR/INGN 201; Ad5CMV-*p53*) were provided by Introgen Therapeutics (Houston, TX). Each vector was a replication-impaired adenovirus in which the E1A and E1B genes required for viral replication were replaced by the transgene coding sequence (firefly luciferase, *Escherichia coli* LacZ, or wild-type human p53, respectively) flanked by the cytomegalovirus (CMV) (Ad-βgal, Adp53) or Rous sarcoma virus (RSV) (AdLuc) promoter and simian virus 40 (SV40) polyadenylation signal. The concentrations of the vectors used for *in vitro* studies and the metastasis study in viral particles (VP) and plaque-forming units (PFU) per milliliter were as follows: Adp53: 1.3×10^{13} VP/ml or 1.2×10^{12} PFU/ml; AdLuc: 5×10^{12} VP/ml or 2×10^{11} PFU/ml. For the subcutaneous tumor model, the concentration of the Adp53 vector stock was 6.55×10^{11} VP/ml or 3.6×10^{10} PFU/ml.

Chemotherapeutic drugs

A 2-mg/ml solution of doxorubicin-HCl for injection (clinical grade; Bedford Laboratories, Bedford, OH) was obtained through local pharmacies.

Cell culture

Cell lines were maintained at 37°C in 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids, pyruvate, and L-glutamine. Fetal bovine serum (heat inactivated at 56°C for 30 min) was added to 10%. All media, additives, and serum were obtained from Irvine Scientific (Santa Ana, CA).

Subcutaneous tumor model

Five- to 8-week-old female nude mice were purchased from Harlan/Sprague Dawley (Indianapolis, IN). Tumors were initiated on the back of each animal by subcutaneous implantation of 10^6 tumor cells in 100 μl of DMEM. Tumor growth was monitored at 2- to 3-day intervals by measuring the length and width of the tumor with a caliper and calculating tumor volume on the basis of the following formula: volume = $(1/2)Lw^2$. When tumors were in the range of 20–60 mm^3 in volume, animals were randomized into treatment groups and treatment was initiated. The first day of treatment was designated as day 1. Tumor growth continued to be monitored by taking measurements at 2- to 3-day intervals. Animals were administered doxorubicin (8 mg/kg) by tail vein injection on days 1 and 15, and Adp53 or AdLuc (3×10^8 PFU per tumor in 100 μl of phosphate-buffered saline [PBS]) on days 2, 4, 7 and 16, 18, 21.

Orthotopic tumor model

An orthotopic model of beast cancer metastasis described by Price and co-workers was used (Price, 1996). Five- to 8-week-old female nude mice were purchased form Harlan/Sprague Dawley. Animals were anesthetized with isofluorane. A small incision was made in the middle of the chest and 2×10^6 cells in 50 μl of DMEM were implanted under the skin in the mammary fat pad. The incision was closed with a surgical staple, and removed about 1 week later. Animals were monitored for

tumor growth by measuring tumors as described above and calculating volumes. As described by Price and colleagues, some 80% of animals acquire lung metastases during the 8 weeks after implantation. By 8 weeks most primary tumors had achieved a size of about 800 mm^3, and were excised at that time. Animals were then randomized into treatment groups of six to eight animals each and were administered treatment (designated day 1). Doxorubicin was administered intravenously via the tail vein on days 1 and 21 (100 μl of a 2-mg/ml solution, or 10 mg/kg). Vector (AdLuc or Adp53) was administered intravenously at 2- to 3-day intervals beginning on day 3 and ending on day 46. A total of 2×10^{10} VP per injection was administered.

Apoptosis assay

An enzyme-linked immunosorbent assay (ELISA) (Boehringer Mannheim, Indianapolis, IN) was used to detect oligonucleosomal fragments released from the nuclei of cells in the early phases of apoptosis, according to the manufacturer instructions and as previously described (Gjerset *et al.*, 1995).

In vitro *assays*

In vitro drug sensitivity was tested by the MTS viability assay based on the bioconversion by metabolically active cells of the tetrazolium compound, MTS (3-[4,5'-dimethylthiazol-2-yl]-5-[3-carboxymethylphenyl]-2-[4-sulfophenyl]-2H-tetrazolium inner salt), into formazan, measured at 490 nm (CellTiter 96 Aqueous; Promega, Madison, WI). Assays are done in triplicate or quadruplicate on at least two independent occasions. Cells plated in 24-well plates at a density of 50–70% were treated with vector (Adp53 or Ad-βgal) at varying multiplicities of infection (generally in the range of 100 PFU per cell for 4 hr to overnight to achieve 50–70% transduction efficiency) and then replated at 1000 cells per well in 96-well plates. Cells were treated the next day with various doses of doxorubicin for 3 hr. Plates were then incubated for 5–6 days, during which time control wells were in exponential growth, followed by a measurement of cell viability. The assay is proportional to the number of living cells in the culture. In some assays, cells were labeled by incubation for 6 hr in the presence of 0.5 μCi of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; Dupont NEN, Boston, MA) per well, followed by harvesting the cells onto glass fiber filters and determining the incorporation of radioactive label by scintillation counting.

Immunohistochemical staining

Lung tissue was fixed in 3.7% buffered formaldehyde, imbedded in paraffin, and sectioned onto slides. Sections were deparaffined by heating to 60°C for 1 hr followed by rehydration by sequential passage in xylene, 100% ethanol, 95% ethanol, and 80% ethanol, followed by H_2O and PBS. Sections were treated with 3% hydrogen peroxide for 5–10 min, and blocked with Superblock (Scytec Laboratories, Logan, UT). Expression of β-galactosidase was detected by placing 200 μl of a 1:1000 dilution of rabbit polyclonal anti-β-galactosidase antibody (5 Prime → 3 Prime, Boulder, CO) on the section and incubating at 4°C for 24 hr. Slides were washed in PBS (3 × 10 min) and incubated with biotinylated goat anti-IgG (Multilink; Biogenex, San Ramon, CA) for 20 min at room temperature, washed in PBS, and incubated with peroxidase-conjugated streptavidin (Biogenex) for 20 min at room temperature. Slides were washed in PBS and incubated with the substrate 3-amino-9-ethylcarbozole (Biogenex) for color development (20 min). Slides were rinsed in deionized H_2O to stop the color developments and mounted with coverslips with aqueous mounting medium (Biogenex).

X-Gal staining of frozen tissue sections

Lung tissue was embedded and frozen in Tissue-Tec embedding medium (Sakura Finetek U.S.A., Torrance, CA). Sections were prepared on slides with a cryostat, and fixed in ethanol–acetone (1:1) at −20°C for 20 min. Slides were then washed with PBS, and sections were circled with a PAP (immunostaining) pen and stained overnight at 37°C with a drop of X-Gal solution in PBS containing 5 m*M* potassium ferrocyanate, 5 m*M* potassium ferricyanate, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 1 mg/ml, dissolved in *N,N*-dimethyl formamide at 100 mg/ml and stored in glass at −20°C). Slides were counterstained with nuclear fast red, dehydrated by sequential passage through ethanol and xylene, and mounted with coverslips, using nonaqueous mounting medium.

RESULTS

Reversible in vitro *growth suppression of MDA-MB-435 cells by Adp53*

Previous studies have demonstrated a suppressive effect of Adp53 on breast cancer cell lines, but have not evaluated the relative contributions of cell cycle arrest or apoptosis. To address this question, the growth-suppressive effects of Adp53 on MDA-MB-435 cells (which express endogenous mutant p53) were evaluated. Cells were treated in 24-well plates with varying amounts of Adp53 or AdLuc for different incubation times. Cells were then replated in 96-well plates in quadruplicate for each treatment condition as described in Materials and Methods, and scored for viability 6 days later by the MTS assay. Viability, as represented as a percentage of the viability of mock-treated control cells, is shown in Fig. 1A. Viability of Adp53-treated cultures decreased with increasing multiplicities of infection and increasing treatment times, in contrast to Ad-Luc-treated cultures, where viabilities remained at 80–100% of control untreated cells, regardless of the multiplicity of infection. Six-day viability of the culture was not completely suppressed by Adp53 even at the highest multiplicity of infection (200 PFU/cell overnight). A shorter term [^{3}H]thymidine assay indicated that addition of Adp53 at 100 PFU/cell resulted in a nearly complete inhibition (90%) of DNA synthesis at 48 hr posttreatment (Fig. 1B), suggesting a high efficiency of gene transduction. The apparent recovery of viable cells at 6 days suggests that some cells underwent a reversible growth arrest rather than cell death, or possibly that nontransduced MDA-MB-435 cells overgrew their transduced counterparts (50–70% transduction efficiency was estimated by X-Gal staining after Ad-βgal transduction at a multiplicity of infection [MOI] of 100 PFU/cell, and is likely to correspond to the percentage of cells expressing high levels of the transgene).

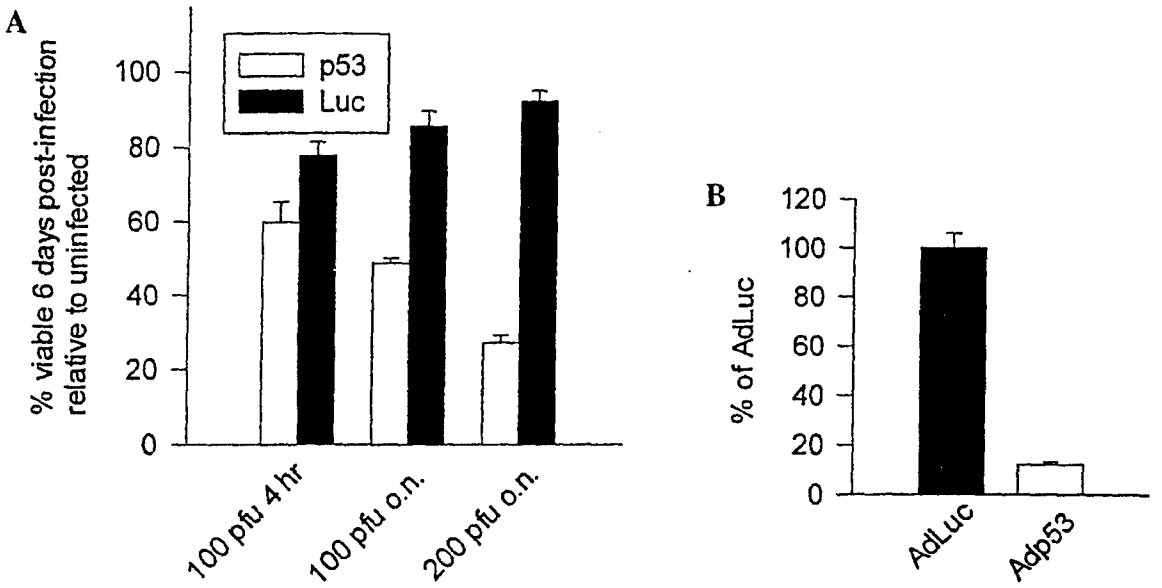

FIG. 1. (A) Viability assay of MDA-MB-435 breast cancer cells 6 days after treatment with the indicated doses of AdLuc (solid columns) or Adp53 (open columns), and expressed as a percentage of untreated cell viability. Cells at 70% confluency were treated and replated in quadruplicate in 96-well plates at 1000 cells per well. Viability was assayed by the MTS assay 6 days from the start of infection. A second assay was performed with similar results. (B) Incorporation of a 4-hr pulse with [^{3}H]thymidine, 48 hr after the start of treatment with AdLuc or Adp53 (100 PFU/cell overnight). Cells were treated and replated in 96-well plates as in (A).

Enhanced doxorubicin-induced apoptosis of MDA-MB-435 cells by p53

Because p53 is known to enhance DNA damage-induced apoptosis and because doxorubixin acts in part through the induction of DNA damage, including intercalation, and covalent adduct formation (Phillips *et al.*, 1989), we asked whether p53 expression would enhance doxorubicin-mediated growth suppression and apoptosis. Figure 2A shows the results of a viability assay performed 6 days after treatment of cells with Adp53 or AdLuc at 100 PFU/cell for 4 hr or overnight, and 5 days after treatment with varying doses of doxorubicin. Results are expressed relative to the 6-day cell viability of the lowest AdLuc treatment (100 PFU/cell; 4 hr) in the absence of doxorubicin and are the averages of quadruplicate wells. As shown in Fig. 2A, Adp53 treatment led to a dose-dependent loss of viability that was enhanced in the presence of doxorubicin. To determine whether this loss of viability correlated with induction of apoptosis, we carried out an apoptosis assay designed to detect the release of oligonucleosomal fragments from the nucleus to the cytoplasm of cells undergoing apoptosis. Cells treated with adenoviral vector at 100 PFU/cell were treated 24 hr later with doxorubicin, and harvested 24 hr after doxorubicin treatment (i.e., 48 hr after vector treatment). At the dose of doxorubicin used (0.1 μM), little apoptosis was observed in the AdLuc + doxorubicin-treated cells. This suggests that the loss of viability we observed with this treatment in Fig. 2A might be a result of nonapoptotic cell death or of transient arrest of the cell cycle. In contrast, apoptosis was readily detectable in cells treated with Adp53 and increased with the addition of 0.1 μM doxorubicin. Overall release of nuclear oligonucleosomes increased some 3- to 5-fold in cells treated with both Adp53 and doxorubicin, and of this, doxorubicin-specific-apoptosis accounted for some 20–40% (Fig. 2B). Thus the apoptosis achieved by the combination of Adp53 + doxorubicin was greater than the sum of what was obtained by Adp53 or doxorubicin (in the presence of AdLuc control vector).

Inhibition of subcutaneous tumor growth by Adp53 in the presence and absence of doxorubicin

A subcutaneous tumor model was used to extend the *in vitro* observations to an *in vivo* model and to provide a point of reference for the subsequent metastasis model. For the subcutaneous model, 10^6 MDA-MB-435 cells were implanted under the skin of the back of female nude mice (Price, 1996). About 10 days postimplantation, or when the average tumor size was about 40 mm^3, animals were randomized into four groups of nine animals each and administered treatment. Doxorubicin was administered intravenously (80 μl of a 2-mg/ml solution, or 8 mg/kg) on days 1 and 15. Vector (Adp53 or AdLuc at 3×10^8 PFU per animal) was administered intratumorally (in 100 μl of PBS) on days 2, 4, and 7, and again on days 16, 18, and 21. Tumors were measured at 2- to 3-day intervals and volumes were calculated and plotted in Fig. 3. We observed the greatest suppression of tumor growth in the Adp53 + doxorubicin treatment group over the entire time course of the study. A one-way analysis of variance (ANOVA) was used to analyze the significance of the difference among data sets on different days, followed by a pairwise Student t test. We found significant differences ($p < 0.05$) between the Adp53 + doxorubicin group and the Adp53 group on days 7, 9, and 11 (following the first cycle of treatment) and again on days 18, 23, 25, and 28 (following the second cycle of treatment). In addition, on days 18, 23, 25, and 28 we also observed significant differences between the Adp53 + doxorubicin group and each of the other three

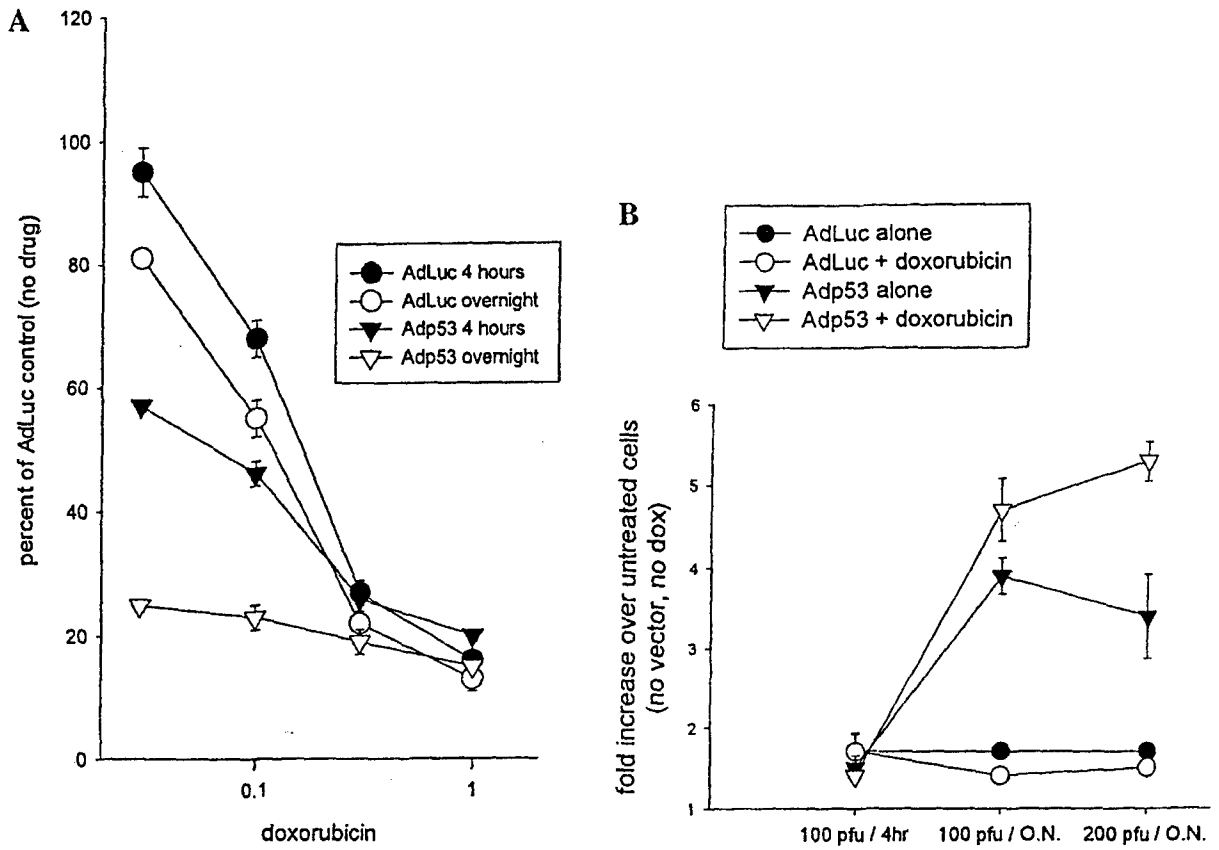

FIG. 2. (A) Viability assay of MDA-MB-435 breast cancer cells treated as in Fig. 1 with Adp53 or AdLuc at 100 PFU/cell for 4 hr or overnight, followed by replating in quadruplicate in 96-well plates. After attachment, cells were treated with the indicated concentrations of doxorubicin (3 hr). Viability was assayed by the MTS assay 6 days from the start of infections. Viability is expressed as a percentage of the viability of cells treated only with AdLuc. (B) Apoptosis assay measuring release of oligonucleosomal fragments from the nucleus. Cells were treated as indicated with AdLuc or Adp53 and cytoplasmic extracts were prepared 48 hr from the start of treatment and assayed for release of oligonucleosomal fragments. Results are expressed as the fold increase relative to untreated cells.

treatment groups ($p < 0.05$). In contrast, on days 18–28, no significant differences were observed between the Ad p53, AdLuc, and AdLuc + doxorubicin treatment groups, indicating that the Adp53 + doxorubicin combination led to increased suppression of tumor growth.

Inhibition of breast cancer metastases by Adp53 and doxorubicin

To determine whether systemically administered Adp53 in combination with doxorubicin improved the outcome for metastatic disease, we employed an orthotopic mammary fat pad breast cancer model in nude mice (Price, 1996). The metastatic clone of MDA-MB-435 cells used in this study gives rise to lung metastases after orthotopic implantation of tumor cells in the mammary fat pad of female nude mice (Fig. 4C, right), providing a model to examine the effects of systemic administration of Adp53. To assess the potential of adenovirus as a systemic gene transfer vector, we injected Adβgal intravenously (10^9 PFU) via the tail vein of mice and examined the transfer of the β-galactosidase gene to lung tissue. Figure 4A shows an X-Gal-stained thin section of the lung of an animal 48 hr after receiving intravenous Ad-βgal, with heavy staining indicating expression of β-galactosidase. Figure 4B shows the results when a paraffin-imbedded, formaldehyde-fixed section of the same lung was immunostained with an anti-β-galactosidase antibody (Fig. 4B, left). A similarly treated section of normal mouse lung was shown for comparison (Fig. 4B, right). This indicates that adenovirus administered intravenously is able to reach the lung and transduce the β-galactosidase gene to lung epithelium, supporting the use of intravenous Adp53 for the treatment of lung metastases.

We then tested the efficacy of Adp53 + doxorubicin combination therapy in eradicating breast cancer lung metastases. Cells (5×10^5) were implanted in the mammary fat pads of female nude mice. At 8 weeks postimplantation primary tumors that had attained a size of 600–900 mm^3 were excised, although these tumors grew back in the second half of the experiment. At 9.5 weeks postimplantation, animals were randomized into treatment groups of seven or eight animals each and treatment was initiated (day 1). Doxorubicin (10 mg/kg, maximum tolerated dose) was administered intravenously on days 1 and 21. Vector was administered at 2- to 3-day intervals for the duration of the experiment (from day 3 and ending on day 46). To equalize potential vector-related toxicities resulting from systemic administration of vector, we administered equivalent

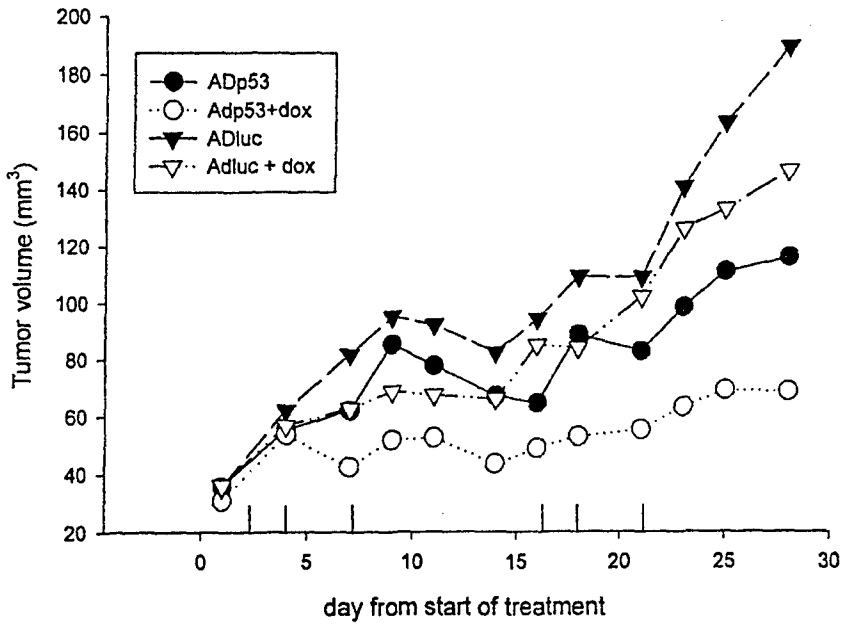

FIG. 3. Subcutaneous model of MDA-MB-435 breast cancer in nude mice. Tumors were established in female nude mice by subcutaneous implantation of 10^6 cells and allowed to grow to an average size of 40 mm^3. Animals with established tumors were randomized into four treatment groups of nine animals each and treatment was initiated (day 1). Each group received intratumoral injection of vector (3×10^8 PFU of AdLuc or Adp53 in 100 μl of PBS) on days 2, 4, and 7 and again on days 16, 18, and 21 (vertical lines). Two groups also received intravenous injection of 100 μl of doxorubicin (8 mg/kg) on days 1 and 15. Tumor volumes were monitored at 2- to 3-day intervals.

amount of vector to control (AdLuc) animals and Adp53 animals (2.5×10^{10} VP of vector per animal per injection in 100 μl of PBS). This corresponded to 3×10^8 PFU in the case of Adp53 and 10^9 PFU in the case of AdLuc.

Metastases were monitored at 15–16 weeks postimplantation (i.e., 7–8 weeks after tumor excision) by killing the animals, removing the lungs, and fixing the lungs in 10% formaldehyde for paraffin embedding and histological analysis. Longitudinal and transverse sections were prepared and analyzed for the incidence of metastases (Table 1). As shown in Table 1, lung metastases were observed in all treatment groups except the group that had been treated with Adp53 + doxorubicin. The Fisher exact probability test was used to examine the significance of the differences in incidence of metastases between the different treatment groups. A significant difference was observed between the Adp53 + doxorubicin and the Adp53 groups ($p < 0.05$), indicating that the Adp53 + doxorubicin combination could reduce the incidence of metastases. A significant difference was also observed between the Adp53 + doxorubicin and the doxorubicin groups ($p < 0.05$). In contrast,

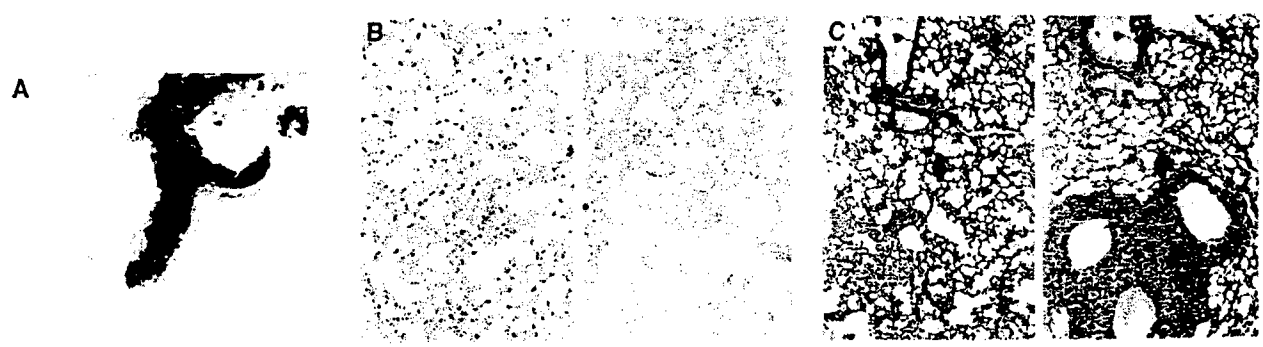

FIG. 4. (A) Frozen section of lung taken from a mouse killed 48 hr after intravenous injection of 10^9 PFU of Adβgal. Section is stained with X-Gal and shows area of staining. (B) Formaldehyde-fixed, paraffin-imbedded section of lung from a mouse killed 48 hr after intravenous injection of Adβgal as in (A), followed by immunohistochemical staining for β-galactosidase (*left*). A similarly treated section of lung from an animal that did not receive vector is also shown (*right*). (C) Formaldehyde-fixed, paraffin-imbedded, and hematoxylin–eosin-stained section of lung of a nude mouse implanted in the mammary fat pad 16 weeks earlier with 5×10^5 MDA-MB-435 cells (*right*), with metastatic mass appearing in the lower half of the panel. Normal mouse lung is shown in the left panel.

TABLE 1. INCIDENCE OF LUNG METASTASES IN NUDE MICE,[a] 16 WEEKS AFTER MAMMARY FAT PAD IMPLANTATION OF 10^5 MDA-MB-435 BREAST CANCER CELLS[b]

Animal group	*No. per group*	*Incidence (histology) (%)*	p *Value*	*Average animal weight (g)*
No treatment	8	38	>0.5	26
Dox	8	50		26
Adp53	8	50	<0.05	26
Adp53 + dox	7	0		24
AdLuc	8	38	>0.5	26
AdLuc + dox	6 (2 deaths)	17		26

[a]Percentage of animals with metastases.

[b]Treatment was initiated 8 weeks post-implantation of tumor cells, and just after excision of the primary tumors. Treatment consisted of vector, 10^{11} viral particles (10^9 PFU) of p53 adenovirus (or AdLuc control) administered 3 times per week by tail vein injection. Some of the animals received doxorubicin intravenously (100 μl of a 2-mg/ml stock, or 10 mg/kg) on days 1 and 21.

no significant differences were observed between the no treatment group and doxorubicin only groups, or between the AdLuc group and the AdLuc + doxorubicin group, as indicated in Table 1. While some micrometastases could have been missed by random sampling, and while we cannot rule out the possibility that micrometastases undetectable at 16 weeks postimplantation may have become detectable at later time points in the Adp53 + doxorubicin group, the extended period of time that elapsed from implantation argues strongly that micrometastases had been suppressed by the treatment. This possibility is also consistent with the *in vitro* studies showing that the combination of Adp53 and doxorubicin promoted apoptosis to a greater extent than Adp53 or doxorubicin alone, thereby reducing the likelihood of reversible cell cycle arrest.

Two deaths that occurred in the AdLuc + doxorubicin treatment group appeared to be disease related. Because these animals were not analyzed for lung metastases, the incidence scored for that group (Table 1) could underrepresent the actual incidence for that group.

Overall toxicity, assessed by weighing the animals at the end of the treatment (Table 1), was minimal, as all groups showed similar average weights. Toxicity to the liver, the organ preferentially targeted by adenovirus in the mouse after intravenous administration, was assessed by histological examination of formaldehyde-fixed liver sections (13 days after the final vector treatment). No pathological diagnoses or any referable observations were made (Fig. 5). Nonspecific splenitis (not shown), typical of animals undergoing procedures, occurred in all animals including controls.

DISCUSSION

This study demonstrates that systemic administration of Adp53 to nude mice bearing mammary fat pad tumors has synergistic antimetastatic activity and minimal toxicity when used in combination with the standard breast cancer chemotherapeutic doxorubicin. When administered locally to subcutaneous tumors, Adp53 as a single agent delivered intratumorally also was effective in reducing tumor growth, and its antitumor effect was enhanced in the presence of doxorubicin, consistent with previous studies (Nielsen *et al.*, 1998; Gurnani *et al.*, 1999). In the metastatic model, however, Adp53 single-agent treatment was not effective in reducing the incidence of lung metastases, possibly because the levels of p53 transgene expression were subthreshold for the induction of apoptosis. Previous studies have demonstrated the efficacy of systemic administration of liposomal p53 DNA complexes in reducing growth of human breast tumors in nude mice (Lesson-Wood *et al.*, 1995; Xu *et al.*, 1997; Liu *et al.*, 1999). In addition, systemic administration of Adp53 has been shown to inhibit metastatic lung cancer in nude mice (Kataoka *et al.*, 1998). However, systemic administration of Adp53 alone or in combination with chemotherapy for the treatment of breast cancer metastases has not previously been evaluated. The present results show significant suppression of the incidence of metastatic growth by Adp53 and doxorubicin even under conditions in which Adp53 and doxorubicin have little or no effect as single agents on metastatic growth. Together with the *in vitro* assays showing that Adp53 treatment of cells enhances doxorubicin-induced apoptosis, the *in vivo* studies suggest a synergistic mechanism of action of the two agents. While the clonal nature of this model system may not accurately reflect the heterogeneity of p53 status of a naturally occurring tumor, we have observed that even wild-type p53-expressing tumor cells can be suppressed by overexpression of ectopic p53 (R.A. Gjerset and T. Tyler, unpublished), suggesting that the suppression we observe in the MDA-MB-435 model system will extend to heterogeneous naturally occurring tumors as well.

Doxorubicin (Adriamycin) is an anthracycline antibiotic, whose cytotoxicity derives from multiple modes of action, including direct damage to DNA. Doxorubicin is able to bind to DNA through intercalation of the planar anthracycline moiety with the DNA double helix. Its polycyclic ring structure and hydroxyl side groups would predispose it to the formation of oxygen free radicals, which could also promote oxidative damage to DNA. Because doxorubicin is lipophilic and can also interact with cellular membranes as well as plasma proteins, it can have an antiangiogenic effect on tumor cell vasculature (Steiner, 1992), as well as a direct effect on tumor cell metabolism. p53 may also have antiangiogenic effects through transcriptional activation of the antiangiogenic factor thrombo-

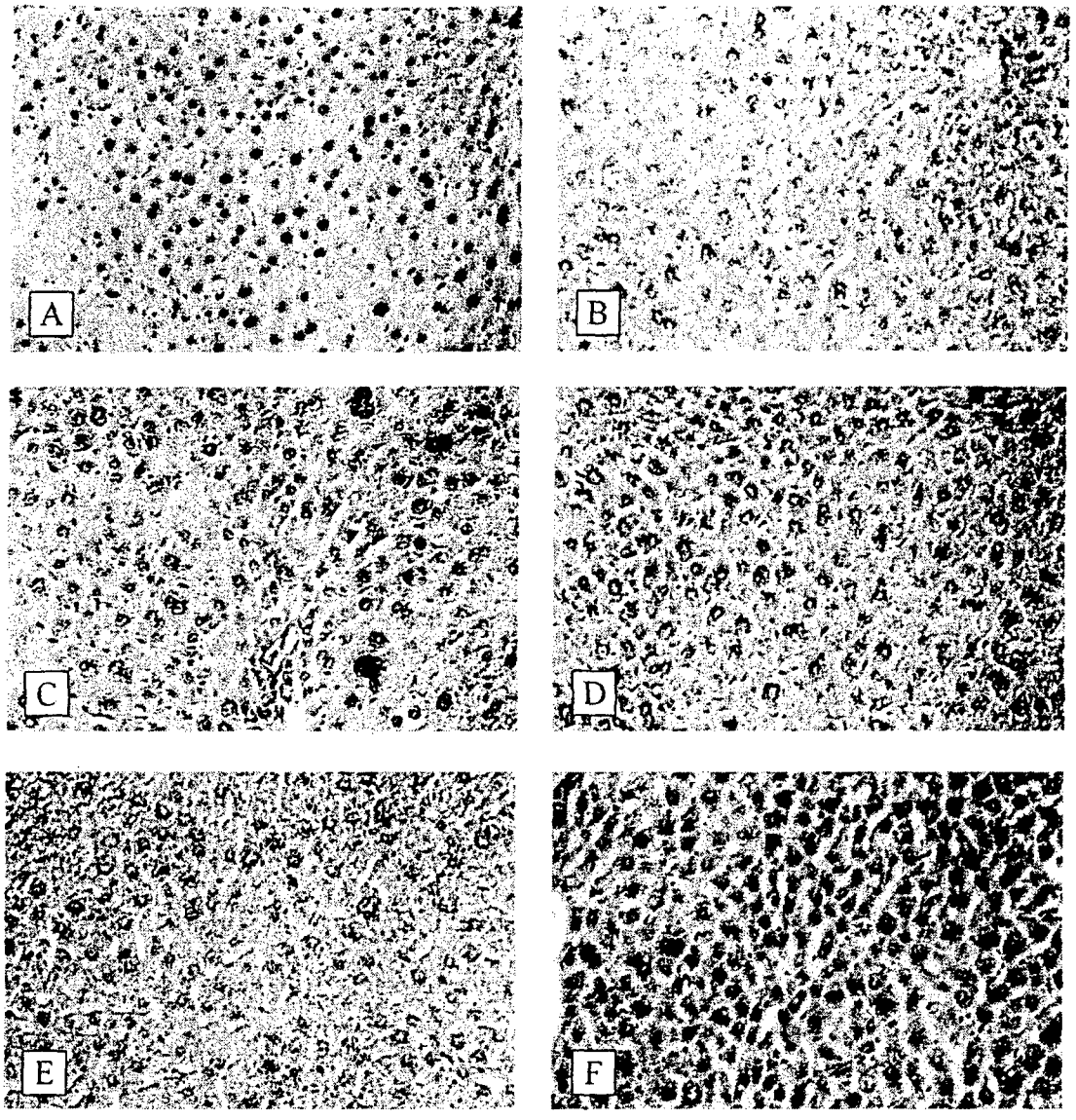

FIG. 5. Formaldehyde-fixed, paraffin-imbedded, and hematoxylin–eosin-stained section of liver taken from mice killed at the termination of the experiment described in Table 1. Sections correspond to the following: (**A**) Adp53-treated animal; (**B**) AdLuc-treated animal; (**C**) Adp53 + doxorubicin-treated animal; (**D**) AdLuc + doxorubicin-treated animal; (**E**) doxorubicin-treated animal; (**F**) control animal (untreated).

spondin, and through downregulation of VEGF (vascular endothelial cell growth factor, and NOS (nitrous oxide synthetase) (Chiarugi *et al.*, 1998). In some cases the marked antitumor activity of p53 gene therapy in animal tumor models despite low efficiencies of gene transfer has correlated with reduced vascularization of wild-type p53-expressing tumors (Nishizaki *et al.*, 1999). The MDA-MB-435 tumor model used in this study was not highly vascularized, as determined by visual examination of tumors, as well as by immunohistochemical staining of formaldehyde-fixed tumor sections for the vascular endothelial cell surface protein PE-CAM (our unpublished data). This suggests that differences in vascularization are unlikely to account for the differences in tumor growth observed between the different treatment groups. Our studies support a model in which p53 and doxorubicin act at least in part on the same DNA damage pathway to lower threshold levels of each needed to induce apoptosis. These results are consistent with studies in which p53 was seen to induce apoptosis in proportion to the extent of DNA damage (Chen *et al.*, 1996). Thus even under conditions in which little growth suppression occurs by p53 alone, a greater than additive combined effect is possible in the presence of doxorubicin.

While the experiments presented here demonstrate the potential of systemic administration of p53 adenovirus in combination with doxorubicin in treating metastatic breast cancer, the clinical application of this approach may be enhanced by steps that enhance serum half-life of the vector or reduce its immunogenicity. Targeted vectors with modified capsids enabling preferential accumulation and internalization into tumor cells may help to reduce immunogenicity and decrease toxicity

(Bilbao *et al.*, 1998). Transcriptional targeting may further enhance the selective expression in tumor cells. PEGylation of adenovirus, in which surface antigens on the virus capsid are covalently modified with polyethylene glycol, has also been effective in protecting the vector from neutralizing antibody (O'Riordan *et al.*, 1999). Administration of adenovirus vectors to humans does not invariably evoke a systemic anti-adenovirus neutralizing antibody, however (Harvey *et al.*, 1999). Furthermore, preexisting humoral and cellular immunity to adenoviral proteins did not preclude gene transfer after intrapleural administration of adenovirus in a phase I trial of suicide gene therapy for mesothelioma (Molnar-Kimber *et al.*, 1998), nor has it abrogated transduction or transgene expression after intratumoral administration (Clayman *et al.*, 1998; Swisher *et al.*, 1999; Nemunaitis *et al.*, 2000). For example, in the phase I clinical trial for non-small cell lung carcinoma (Swisher *et al.*, 1999), and the phase I clinical trial for head and neck cancer (Clayman *et al.*, 1998), patients received, respectively, one injection per month, and six injections over a 2-week period repeated monthly, and some patients received treatment for as long as 6–7 months. Systemic circulation of Adp53 was observed in patients after intratumoral injection (Nemunaitis *et al.*, 2000). In both studies, investigators demonstrated gene transduction and transgene expression in biopsy material from patients with elevated antibody titers. Thus the immunogenicity of adenovirus may not pose an absolute barrier to systemic administration.

It is becoming increasingly evident that the therapy-resistance phenotype characteristics of tumor cells is closely linked to the underlying mechanism of cancer. Many of the gene changes known to promote cellular transformation and tumor progression, including loss of wild-type p53 function, activation of the *ras* oncogene, and overexpression of Her-2/*neu*, also contribute to drug resistance (McKenna *et al.*, 1990; Tsai *et al.*, 1996; O'Connor *et al.*, 1997). In this study we focused on the p53 tumor suppressor because the high frequency with which it is lost in cancer points to a central role in tumor suppression and therapy responsiveness. The fact that loss of p53 is also associated with increased genome instability (Donehower *et al.*, 1995) suggests that loss of p53 renders cells permissive for genome-destabilizing events involving strand breaks. Thus failure of p53-mediated DNA damage-induced apoptosis promotes both survival of tumor cells with unstable genomes, and resistance to DNA-damaging chemotherapeutic drugs.

These data are particularly relevant for metastatic disease, where therapy resistance is the primary reason for treatment failure. Reversal of resistance may be possible through the application of systemic biological approaches such as p53 gene therapy, which target the DNA damage response, and provide an opportunity to fully exploit the potential of conventional therapies.

ACKNOWLEDGMENTS

This work was supported by grants (R.A.G.) from the Department of Defense Breast Cancer Research Program (DAMD17-96-1-6038), the National Cancer Institute (CA69546), and Introgen Therapeutics, Inc. We thank Janet Price, Ph.D. (M.D. Anderson Cancer Research Center) for providing us with the metastatic clone of MDA-MB-435 cells. We thank Dr. Dan Mercola, M.D., Ph.D., F.C.A.P. (Sidney Kimmel Cancer Center) for microscopic analysis of tissues.

REFERENCES

BARTEK, J., IGGO, R., GANNON, J., and LANE, J.P. (1990). Genetic and immunochemical analysis of mutant p53 in human breast cancer lines. Oncogene **5**, 893–899.

BILBAO, G., GOMEZ-NAVARRO, J., and CURIEL, D.T. (1998). Targeted adenoviral vectors for cancer gene therapy. Adv. Exp. Med. Biol. **451**, 365–374.

CHEN, X., KO, L.J., JAYARAMAN, L., and PRIVES, C. (1996). p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev. **10**, 2438–2451.

CHIARUGI, V., MAGNELLI, L., and GALLO, O. (1998). Cox-2, iNOS and p53 as play-makers of tumor angiogenesis [review]. Int. J. Mol. Med. **2**, 715–719.

CLAYMAN, G.L., EL-NAGGAR, A.K., LIPPMAN, S.M., HENDERSON, Y.C., FREDERICK, M., MERRITT, J.A., ZUMSTEIN, L.A., TIMMONS, T.M., LIU, T.J., GINSBERG, L., ROTH, J.A., HONG, W.K., BRUSO, P., and GOEPFERT, H. (1998). Adenovirus-mediated p53 gene transfer in patients with advanced head and neck squamous cell carcinoma. J. Clin. Oncol. **16**, 2221–2232.

COLES, C., CONDIE, A., CHETTY, U., STEEL, C.M., EVANS, A.J., and PROSSER, J. (1992). p53 mutations in breast cancer. Cancer Res. **52**, 5291–5298.

CUNY, M., KRAMAR, A., COURJAL, F., JOHANNSDOTTIR, V., IACOPETTA, B., FONTAINE, H., GRENIER, J., CULINE, S., and THEILLET, C. (2000). Relating genotype and phenotype in breast cancer: An analysis of the prognostic significance of amplification at eight different genes or loci and p53 mutations. Cancer Res. **60**, 1077–1083.

DONEHOWER, L.A., GODLEY, L.A., ALDAZ, C.M., PYLE, R., SHI, Y.-P., PINKEL, D., GRAY, J., BRADELY, A., MEDINA, D., and VARMUS, H.E. (1995). Deficiency of p53 accelerates mammary tumorigenesis in wnt-1 transgenic mice and promotes chromosomal instability. Genes Dev. **9**, 882–895.

DORIGO, O., TURLA, S.T., LEBEDEVA, S., and GJERSET, R.A. (1998). Sensitization of rat glioblastoma multiforme to cisplatin *in vivo* following restoration of wild-type p53 function. J. Neurosurg. **3**, 535–540.

EASTMAN, A., and RIGAS, J.R. (1999). Modulation of apoptosis signaling pathways and cell cycle regulation. Semin. Oncol. **5**(Suppl. 16), 7–16.

FERREIRA, C.G., TOLIS, C., and GIACCONE, G. (1999). p53 and chemosensitivity. Ann. Oncol. **10**, 1011–1021.

GJERSET, R.A., and MERCOLA, D. (2000). Sensitization of tumors to chemotherapy through gene therapy. Adv. Exp. Med. Biol. **465**, 273–291.

GJERSET, R.A., TURLA, S.T., SOBOL, R.E., SCALISE, J.J., MERCOLA, D., COLLINS, H., and HOPKINS, P.J. (1995). Use of wild-type p53 to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. Mol. Carcinog. **4**, 275–285.

GURNANI, M., LIPARI, P., DELL, J., SHI, B., and NIELSEN, L.L. (1999). Adenovirus-mediated p53 gene therapy has greater efficacy when combined with chemotherapy against human head and neck, ovarian, prostate, and breast cancer. Cancer Chemother. Pharmacol. **44**, 143–151.

HARVEY, B.G., HACKETT, N.R., EL-SAWY, T., ROSENGART, T.K., HIRSCHOWITZ, E.A., LIEBERMAN, M.D., LESSER, M.L., and CRYSTAL, R.G. (1999). Variability of human systemic humoral immune responses to adenovirus gene transfer vectors administered to different organs. J. Virol. **73**, 6729–6742.

HENDERSIN, I.C., HARRIS, J.R., KINNE, D.W., and HELLMAN, S. (1998). Cancer of the breast. In *Cancers: Principles and Practice of*

Oncology, 3rd Ed. V.T. DeVita, S. Hellman, and S.A. Rosenberg, eds. (Lippincott, Philadelphia, PA) pp. 1197–1268.

KATAOKA, M., SCHUMACHER, G., CRISTIANO, R.J., ATKINSON, E.N., ROTH, J.A., and MUKHOPADHYAY, T. (1998). An agent that increases tumor suppressor transgene product coupled with systemic transgene delivery inhibits growth of metastatic lung cancer *in vivo*. Cancer Res. **58,** 4761–4765.

LAKIN, N.D., and JACKSON, S.P. (1999). Regulation of p53 in response to DNA damage. Oncogene **18,** 7644–7655.

LANE, D.P. (1999). Exploiting the p53 pathway for cancer diagnosis and therapy. Br. J. Cancer. **80**(Suppl. 1), 1–5.

LESSON-WOOD, L.A., KIM, W.H., and KLEINMAN, H.K. (1995). Systemic gene therapy with p53 reduces growth and metastases of a malignant human breast cancer in nude mice. Hum. Gene Ther. **6,** 395–405.

LIU, Y., THOR, A., and SHTIVELMAN, E. (1999). Systemic gene delivery expands the repertoire of effective antiangiogenic agents. J. Biol. Chem. **274,** 13338–13344.

LOTEM, J., and SACHS, L. (1993). Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents. Blood **82,** 1092–1096.

LOWE, S.W., RULEY, H.E., JACKS, T., and HOUSMAN, D.E. (1993). p53-mediated apoptosis modulates the cytotoxicity of anticancer agents. Cell **74,** 957–967.

MAY, P., and MAY, E. (1999). Twenty years of p53 research: structural and functional aspects of the p53 protein. Oncogene **53,** 7621–7637.

McKENNA, W.G., WEISS, M.C., ENDRICH, B., LING, C.C., BAKANAUSKAS, V.J., KELSTEN, M.L., and MUSCHEL, R.J. (1990). Synergistic effect of the c-myc oncogene with H-ras on radioresistance. Cancer Res. **50,** 97–102.

MOLNAR-KIMBER, K.L., STERMAN, D.H., CHANG, M., KANG, E.H., ELBASH, M., LANUTI, M., ELSHAMI, A., GELFAND, K., WILSON, J.M., KAISER, L.R., and ALBELDA, S.M. (1998). Impact of preexisting and induced humoral and cellular immune responses in an adenovirus-based gene therapy phase I clinical trial for localized mesothelioma. Hum. Gene Ther. **9,** 2121–2133.

NEMUNAITIS, J., SWISHER, S.G., TIMMONS, T., CONNORS, D., MACK, M., DOERKSEN, L., WEILL, D., WAIT, J., LAWRENCE, D.D., KEMP, B.L., FOSSELLA, F., GLISSON, B.S., HONG, W.K., KHURI, F.R., KURIE, J.M., LEE, J.J., LEE, J.S., NGUYEN, D.M., NESBITT, J.C., PEREZ-SOLER, R., PISTERS, K.M., PUTNAM, J.B., RICHLI, W.R., SHIN, D.M., WALSH, G.L., MERRITT, J., and ROTH, J. (2000). Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. J. Clin. Oncol. **18,** 609–622.

NIELSEN, L.L., LIPARI, P., DELL, J., GURNANI, M., and HAJIAN, G. (1998). Adenovirus-mediated p53 gene therapy and paclitaxel have synergistic efficacy in models of human head and neck, ovarian, prostate, and breast cancer. Clin. Cancer Res. **4,** 835–846.

NIGRO, J.M., BAKER, S.J., PREISINGER, A.C., JESSUP, J.M., HOSTETTER, R., CLEARY, K., BIGNER, S.H., DAVIDSON, N., BAYLIN, S., and DEVILEE, P. (1989). Mutations in the p53 gene occur in diverse human tumor types. Nature **342,** 705–708.

NISHIZAKI, M., FUJIWARAM T., TANIDA, T., HIZUTA, A., NISHIMORI, H., TOKINO, T., NAKAMURA, Y., BOUVET, M., ROTH, J.A., and TANAKA, N. (1999). Recombinant adenovirus expressing wild-type p53 is antiangiogenic: A proposed mechanism for bystander effect. Clin. Cancer Res. **5,** 1015–1023.

O'CONNOR, P.M., JACKMAN, J., BAE, I., MYERS, T.G., FAN, S., MUTOH, M., SCUDIERO, D.A., MONKS, A., SAUSVILLE, E.A., WEINSTEIN, J.N., FRIEND, S., FORNACE, A.J., JR., and KOHN, K.W. (1997). Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anti-cancer drug screen and correlations with the growth-inhibitory potency of 123 anti-cancer agents. Cancer Res. **57,** 4285–4300.

O'RIORDAN, C.R., LACHAPELLE, A., DELGADO, C., PARKES, V., WADSWORTH, S.C., SMITH, A.E., and FRANCIS, G.E. (1999). PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody *in vitro* and *in vivo*. Hum. Gene Ther. **10,** 1349–1358.

PHAROAH, P.D., DAY, N.E., and CALDAS, C. (1999). Somatic mutations in the p53 gene and prognosis in breast cancer: A meta-analysis. Br. J. Cancer **80,** 1968–1973.

PHILLIPS, D.R., WHITE, R.J., and CULLINANE, C. (1989). DNA sequence-specific adducts of adriamycin and mitomycin C. FEBS Lett. **246,** 233–240.

POWELL, B., SOONG, R., IACOPETTA, B., SESHADRI, R., and SMITH, D.R. (2000). Prognostic significance of mutations to different structural and functional regions of the p53 gene in breast cancer. Clin. Cancer Res. **6,** 443–451.

PRICE, J.E. (1996). Metastasis from human breast cancer cell lines. Breast Cancer Res. Treat. **39,** 93–102.

ROTH, J.A., SWISHER, S.G., and MEYN, R.E. (1999). p53 tumor suppressor gene therapy for cancer. Oncology (Huntingt) **10**(Suppl. 5), 148–154.

SETH, P., KATYOSE, Y., and RAKKAR, A.N.S. (1999). Adenoviral vectors for cancer gene therapy. In *Adenoviruses: Basic Biology to Gene Therapy*. P. Seth, ed. (R.G. Landes, Austin, TX) pp 103–120.

SINGH, S., SIMON, M., MEYBOHM, I., JANTKE, I., JONAT, W., MAASS, H., and GOEDDE, H.W. (1993). Human breast cancer: Frequent p53 allele loss and protein overexpression. Hum. Genet. **90,** 635–640.

STEINER, R. (1992). In *Angiogenesis: Key Principles—Science, Technology and Medicine*. R. Steiner, P.B. Weisz, and R. Langer, eds. (Birkhauser, Basel, Switzerland) pp. 449–454.

SWISHER, S.G., ROTH, J.A., NEMUNAITIS, J., LAWRENCE, D.D., KEMP, B.L., CARRASCO, C.H., CONNORS, D.G., EL-NAGGAR, A.K., FOSSELLA, F., GLISSON, B.S., HONG, W.K., KHURI, F.R., KURIE, J.M., LEE, J.J., LEE, J.S., MACK, M., MERRITT, J.A., NGUYEN, D.M., NESBITT, J.C., PEREZ-SOLER, R., PISTERS, K.M., PUTNAM, J.B. JR., RICHLI, W.R., SAVIN, M., and WAUGH, M.K. (1999). Adenovirus-mediated p53 gene transfer in advanced non-small-cell lung cancer. J. Natl. Cancer Inst. **91,** 763–771.

TSAI, C.M., CHANG, K.T., WU, L.H., CHEN, J.Y., GAZDAR, A.F., MITSUDOMI, T., CHEN, M.H., and PERNG, R.P. (1996). Correlations between intrinsic chemoresistance and *HER-2/neu* gene expression, p53 gene mutations, and cell proliferation characteristics in non-small cell lung cancer cell lines. Cancer Res. **56,** 206–209.

XU, M., KUMAR, D., SRINIVAS, S., DETOLLA, L.J., YU, S.F., STASS, S.A., and MIXSON, A.J. (1997). Parenteral gene therapy with p53 inhibits human breast tumors *in vivo* through a bystander mechanism without evidence of toxicity. Hum. Gene Ther. **8,** 177–185.

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Received for publication October 16, 2000; accepted after revision February 22, 2001.

Treatment Resistance, Apoptosis, and *p53* Tumor Suppressor Gene Therapy

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GLOSSARY

apoptosis a gene-activated process resulting in physiological or "programmed" cell death. Apoptosis was initially defined by characteristic morphological criteria, including cell shrinkage, membrane "blebbing," and nuclear fragmentation. Anticancer agents, including radiation, kill cells by inducing apoptosis.

bax a member of the *bcl2* gene family and an important component in the regulation of apoptosis. In association with *bcl2*, *bax* forms a heterodimer that blocks the anti-apoptotic activity of *bcl2*.

bcl2 a cellular gene whose expression inhibits apoptosis. An oncogene in follicular B cell lymphoma where failure of apoptosis is due to upregulated expression of the *bcl2* gene as a result of a chromosomal translocation t(18;14) that places the *bcl2* gene under the control of the immunoglobulin promoter.

c-*fos* a cellular gene whose gene product is a component of the AP-1 transcription factor involved in signal transduction pathways for DNA repair and cell proliferation.

cyclin-dependent kinases (CDKs) proteins required for cell cycle progression that regulate cellular proliferation.

GADD45 an inducible DNA repair component of the *p53*-mediated response to DNA damage. *p53* induces the expression of *GADD45*, whose gene product associates with proliferating cell nuclear antigen (PCNA), a protein involved in DNA repair.

multiple drug resistance gene (MDR1) a gene that encodes an energy-dependent efflux pump (P-glycoprotein) for hydrophobic cytotoxic drugs such as adriamycin, vincristine, etoposide and taxol. Expression of P-glycoprotein is elevated in many tumor cell lines and surgical specimens.

p16 a cell cycle regulatory protein, whose loss is a feature of some cancers and most cultured tumor cell lines. p16 appears to specifically inhibit cyclin-dependent kinase 4, required for progression into S phase.

p21(waf-1, Cip1, Sdi 1) a gene product that is an inhibitor of several cyclin-dependent kinases required for cell cycle progression and is a potent cell cycle suppressor by itself. As with *GADD45*, *p53* induces the expression of *p21* which also associates with PCNA.

p53 a tumor suppressor gene which is abnormal in approximately half of all cancers. A central regulatory gene encoding a protein involved in cell proliferation, DNA repair, and apoptosis.

proliferating cell nuclear antigen (PCNA) a nuclear protein associated with CDK complexes and involved in DNA replication and repair. PCNA associates with the *p53*-induced proteins GADD45 and p21.

wild type a genetic term designating the normal or nonmutated form of a gene or gene product.

Traditional cancer therapy has relied heavily on chemotherapy and radiation treatment for decades. Despite the introduction of new and more effective drugs and radiotherapy techniques, many cancers that are not completely removed by surgical resection remain incurable. The major reason for treatment failure is the acquisition of therapy resistance. Tumors which initially respond to treatment, eventually undergo genetic changes which confer therapy resistance. Several mechanisms of chemotherapy resistance have been identified, including decreased drug uptake, metabolic detoxification, and increased drug extrusion. In addition, drugs and radiation which damage DNA can also lose effectiveness as cells upregulate DNA repair mechanisms. However, virtually all anticancer agents kill cells by collaborating with the cell's own suicide program. This program, known as programmed cell death, or apoptosis, must be functioning in order for the cell to be sensitive to treatment. Thus, another important mechanism that contributes to therapy resistance is the loss of the metabolic pathways responsible for triggering apoptosis. In this regard, the study of the molecular mechanisms responsible for apoptosis has become an area of intense basic and applied research interest. This article reviews the process of apoptosis and describes how disruption of apoptosis contributes to tumorigenesis and therapy resistance. This article also discusses the central role of the tumor suppressor *p53* in apoptosis, and how loss of normal (or wild-type) *p53* function inhibits apoptotic pathways and results in drug and radiation resistance. Novel cancer treatments based on *p53* gene transfer will also be considered.

I. PROGRAMMED CELL DEATH (APOPTOSIS)

Apoptosis was initially defined by characteristic morphological criteria, including cell shrinkage, membrane "blebbing," and nuclear fragmentation. Programmed cell death has long been recognized as an integral component of many normal biological processes, such as embryonic development and thymic selection of the immune repertoire. Although still incompletely defined at the biochemical level, apoptosis is a gene-activated process involving cellular metabolism. In some cases, apoptosis can be inhibited by metabolic inhibitors such as cyclohexamide. The sequence of metabolic events that occurs in apoptosis includes elevation of cellular calcium, activation of calcium-dependent endonucleases, fragmentation of genomic DNA (often to multiples of the 200-bp nucleosome repeat unit), expression of new cell surface antigens, and finally engulfment of the apoptotic cell by neighboring cells such as tissue phagocytes. Apoptosis represents a form of physiological cell death which eliminates cells marked for destruction, without causing damage to the surrounding tissue.

This is in contrast to the process of cell death termed *necrosis*, which can result in considerable damage to surrounding tissue due to the induction of an inflammatory response following cell rupture. Necrosis is not genetically determined, but can occur in any cell that has been exposed to toxic environmental conditions. Rather than using cell metabolic pathways, necrosis involves a rapid and general collapse of cellular metabolism accompanied by a depletion of ATP levels. Necrotic cells lose membrane integrity and eventually rupture, releasing their contents into the surrounding tissue.

II. CANCER PATHOGENESIS AND FAILURE OF APOPTOSIS

In addition to the processes of embryonic development and thymic selection, apoptosis is believed to

play an important role in protecting multicellular organisms from cancer. Genetic abnormalities associated with cancer may lead to failure of apoptosis. It became evident with the identification of the oncoprotein *bcl2* that disruption of cell death pathways could play as important a role in cancer pathogenesis as deregulation of cellular proliferation. For example, the malignant B cells of patients with follicular lymphoma accumulate in part because they fail to die by apoptosis. Failure of apoptosis in these cells is due to upregulated expression of the *bcl2* gene product which is overproduced as a result of a chromosomal translocation t(18;14) that places the *bcl2* gene under the control of the immunoglobulin promoter. Similarly, transgenic mice that overexpress *bcl2* in their B cells suffer from benign follicular hyperplasia, in which nonproliferating B cells accumulate. With additional genetic alterations, the benign hyperplasia can evolve into a malignant lymphoma.

Disruption of apoptosis is probably integral to all or most types of cancer, as studies on the tumor suppressor *p53* suggest. Unlike *bcl2*, for which deregulation occurs in a restricted array of tumors, *p53* alterations are widespread in human cancer, affecting at least half of all cancers. Normal *p53* is known to block the proliferation of tumor cells and to trigger apoptosis in some instances. Several recent studies have shown that *p53* is essential for triggering apoptosis in response to DNA damage. It is known that cells expressing wild-type (normal) *p53* undergo a transient cell cycle arrest just prior to commencing replicative DNA synthesis. This does not occur in cells with aberrant *p53* function. This has led to a model of *p53* function as a guardian of the cellular genome. In this model, *p53* blocks the cell cycle in response to DNA damage which provides time for cellular DNA repair. In the event that repair is unsuccessful, *p53* then triggers apoptosis. In this manner, cells harboring potentially neoplastic alterations in their genome would be eliminated (Fig. 1).

This model helps to explain the strong selection pressure to lose *p53* function in tumors. One of the hallmarks of cancer is karyotypic instability, a feature that may be the driving force for cancer progression. Loss of *p53* function may favor the outgrowth of karyotypically unstable cancer cells by removing the trigger for apoptosis that would eliminate cells with unstable genomes. Transgenic mice which lack *p53* show a

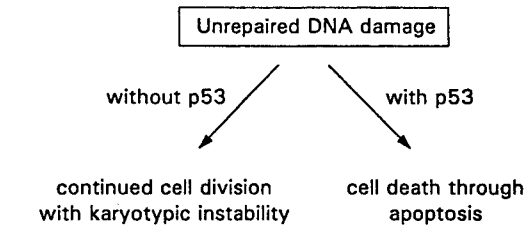

FIGURE 1 Guardian role of *p53*. Scheme illustrating the guardian role of *p53* in eliminating cells with unstable genomes.

dramatically increased susceptibility to cancer with virtually 100% developing one or several tumors before the age of 6 months. Similarly, the Li–Fraumeni familial cancer syndrome in humans, also characterized by a dramatic increased susceptibility to cancer, is associated with the inheritance of a mutant *p53* allele. Normal cells in these patients continue to express both the normal *p53* allele and the mutant allele. The tumor cells, however, lose expression of the normal allele, along with the guardian function that it provides.

III. THERAPY RESISTANCE, FAILURE OF APOPTOSIS, AND *p53* ABNORMALITIES

In addition to its involvement in cancer pathogenesis, loss of *p53* function contributes to therapy resistance. Anticancer agents, including radiation, kill cells by apoptosis, and several lines of evidence have implicated *p53* as an essential element in the process. Normal hematopoietic cells and fibroblasts from *p53*-deficient transgenic mice are resistant to killing by a variety of different anticancer drugs and radiation. In contrast, cells from the parental strain of mice which express wild-type (normal) *p53* are readily killed by these treatments. Furthermore, oncogene-transformed fibroblasts from *p53*-deficient transgenic mice form tumors *in vivo* which are more resistant to anticancer drugs and radiation. In human cancer cells, a role for *p53* in therapy sensitivity is suggested by a recent study of Burkitt's lymphoma cell lines where loss of wild-type *p53* function was associated with radiation resistance. In another recent study, the transfer of wild-type *p53* genes into *p53*-negative non-small cell lung cancer (nsclc) cells restored cisplatin sensitivity in culture. In a nude mouse model of nsclc

tumors, *in vivo* gene transfer of wild-type *p53* into subcutaneous tumors resulted in enhanced *in vivo* sensitivity to cisplatin. These studies suggested that *p53* plays an important role in treatment sensitivity.

Wild-type *p53* gene transfer can also enhance the treatment sensitivity of tumor cells expressing endogenous mutant *p53*, a more common type of *p53* abnormality in human cancer than *p53* deletion. This is important because expression of wild-type *p53* may not by itself be sufficient to suppress tumor cell growth. The bar graph in Fig. 2 illustrates this point for two clones of the same T98G glioblastoma cell line that differ only in the expression of wild-type *p53*. Clone a expresses only mutant *p53* (codon $237^{Met \rightarrow Ile}$). Clone b expresses both mutant and wild-type *p53* (introduced by gene transfer), but the level of expression of wild-type *p53* is low enough to be compatible with cell proliferation. However, in the presence of the DNA-damaging drug cisplatin (at 30 μM), the viability of clone a and clone b differs significantly. Sensitivity to cisplatin is markedly enhanced in clone b cells that express wild-type *p53* and are able to trigger their own cell death program. Enhancement of cisplatin sensitivity also occurs in small cell lung carcinoma cells that express a different mutation in *p53* (codon 246). Therapy sensitivity was correlated with the induction of apoptosis in these studies. Thus *p53* may be involved as a direct sensor of various forms of DNA damage, including damage arising from the intrinsic genomic instability of the cancer cell, as well as damage induced by radiation or by chemotherapeutic drugs such as cisplatin. Although high levels of DNA damage may still lead to cell death through a *p53*-independent mechanism, the presence of *p53* would be expected to lower the threshold of damage required to trigger apoptosis, and this may explain why *p53* enhances therapy sensitivity. Taken together, these observations provide a basis for developing novel forms of cancer therapy where *p53* gene transfer is utilized to increase the efficacy of other treatment modalities. These initial results suggest a dominant effect of wild-type *p53* over the mutant *p53* and imply that wild-type *p53* gene transfer may find future clinical application in combination with conventional chemotherapy and radiation.

Further studies with a larger number of tumor cells will be necessary to extend these observations to the broader spectrum of known *p53* mutations. *p53* is a complex protein involved in a variety of interactions and functions, including oligermization with itself, binding to other cellular and viral proteins, and activating transcription through sequence-specific DNA binding. Although the studies described earlier suggest that *p53* gene transfer may have widespread applicability to a variety of human cancers, the multifunctional nature of *p53* precludes generalizations about its effect in cancers expressing specific mutations. A wide array of mutations occurs in human cancer, and some of these may play differing roles in tumorigenesis and therapy resistance. As indicated in Fig. 3, *p53* consists of 393 amino acids with most mutations occurring in the central highly conserved region which interacts in a sequence-specific manner with DNA. Hot spots for mutation occur at codons 175 (Arg), 248 (Arg), and 273 (Arg), as indicated by arrows in the figure. Two of these arginines (248 and 273) form

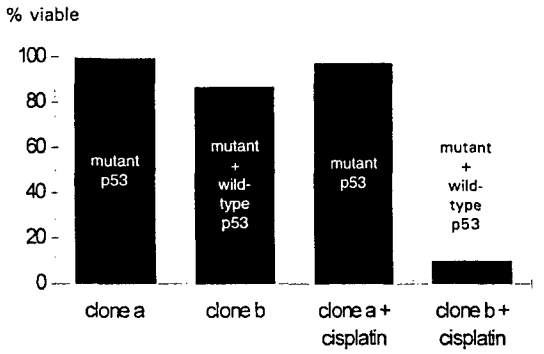

FIGURE 2 Cisplatin sensitivity and wild-type *p53*. Bars indicate relative 7-day growth of two clones of T98G glioblastoma cells differing in wild-type *p53* expression. Clone a expresses only mutant *p53* (codon $237^{Met \rightarrow Ile}$) whereas clone b expresses mutant and wild-type *p53*. In the absence of cisplatin, both clones grow with similar growth kinetics. However, clone b shows a markedly enhanced sensitivity to cisplatin.

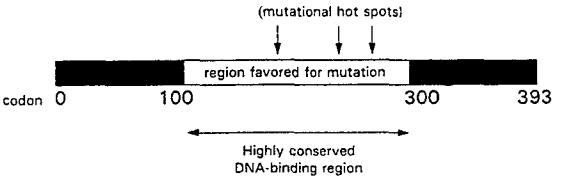

FIGURE 3 Location of *p53* mutations. The scheme shows the 393 codon amino acid sequence of *p53*. The central highly conserved region that interacts with DNA is the most commonly mutated region in human cancer. Hot spots for mutation are indicated by arrows and occur at arginines 175, 248, and 273.

direct electrostatic interactions with the DNA phosphate backbone and are therefore critical for anchoring the *p53* protein to DNA. The third arginine (175) is important in maintaining the structural integrity of the DNA-binding domain and would indirectly affect DNA binding through its effect on protein structure. Further studies will be needed to determine whether wild-type *p53* can also enhance therapy sensitivity to tumors expressing these common types of mutation.

IV. THERAPEUTIC APPLICATIONS

These observations concerning the loss of *p53* function in tumorigeneis and treatment resistance have led to the development of Phase I clinical trials of *p53* gene transfer. *p53* gene therapy clinical trials for lung, head and neck, hepatocellular, and colorectal carcinomas have been submitted to regulatory agencies for approval (Table I). In the head and neck and lung cancer protocols, *p53* viral vectors will be injected directly into tumor nodules while hepatocellular carcinomas and liver metastases of colorectal carcinoma will be treated by hepatic artery infusion of a *p53* adenoviral vector. The safety of *p53* gene transfer will be assessed in these studies which will pave the way for a new generation of cancer therapeutics based on correction of the genetic abnormalities responsible for tumor pathogenesis and treatment resistance. The observation that wild-type *p53* can enhance the sensitivity of tumor cells to chemotherapy and radiation is significant in the context of *p53* gene therapy, which aims toward the suppression of cell proliferation to achieve antitumor effects. Since the expression of wild-type *p53* in tumors may result merely in reversible G_1 arrest, sustained transgene expression would be required for complete tumor suppression, a requirement which would limit the usefulness of *p53* gene therapy with current vector systems. However, the studies reviewed earlier suggest that transient expression of wild-type *p53* may be effective when combined with traditional therapeutic approaches by sensitizing the cancer cell to the DNA-damaging effects of radiation and chemotherapy.

V. FUTURE DIRECTIONS: *p53* GENE INTERACTIONS

The central regulatory role of *p53* is underscored by the variety of cellular processes in which it has been implicated, including cell proliferation, cell differentiation, programmed cell death (apoptosis), cellular senescence, DNA repair, and transcriptional transactivation. The genes involved in these processes could also play critical roles in the *p53*-mediated response to DNA damage, and eventually it may be possible to target these steps, either alone or in combination

TABLE I
Clinical Protocols of *p53* Tumor Suppressor Gene Therapy

Tumor	Protocol title	Principal investigator	Institution/country
Lung non-small cell	Clinical Protocol for Modification of Oncogene and Tumor Suppressor Gene Expression in Non-Small Cell Lung Cancer	J. A. Roth	MD Anderson Cancer Center/ Houston, TX
Lung non-small cell	Clinical Protocol for Modification of Tumor Suppressor Gene Expression and Induction of Apoptosis in Non-Small Cell Lung Cancer (NCSLC) with an Adenovirus Vector Expressing Wild-Type *p53* and Cisplatin	J. A. Roth	MD Anderson Cancer Center/ Houston, TX
Head and neck squamous cell carcinoma	Clinical Protocol for Modification of Tumor Suppressor Gene Expression in Head and Neck Squamous Cell Carcinoma with an Adenovirus Vector Expressing Wild-Type *p53*	G. L. Clayman	MD Anderson Cancer Center/ Houston, TX
Colorectal liver metastases hepatocellular	Gene Therapy of Primary and Metastatic Malignant Tumors of the Liver Using ACN53 via Hepatic Artery Infusion: A Phase I Study	A. P. Venook and R. S. Warren	University of California/San Francisco, CA

with *p53*, to achieve better enhancement of treatment sensitivity. For example, wild-type *p53* downregulates the expression of c-*fos*, a factor which is critical to DNA repair and which is known by itself to affect sensitivity to cisplatin. Since downregulation of c-*fos* has been shown to reestablish drug sensitivity in tumor lines selected for drug resistance, *p53* may also increase drug sensitivity through its affect on c-*fos*. Since *p53* is rapidly degraded in normal cells, but has a dramatically extended half-life in tumor cells, the effect of *p53* on c-*fos* may be preferentially inhibitory to tumor cells. In addition, wild-type *p53* may lead to reduced expression of the multidrug resistance gene (MDR1) whose gene product, P-glycoprotein, is involved in an energy-dependent efflux pump for hydrophobic cytotoxic drugs (such as adriamycin, vincristine, etoposide, and taxol). Expression of P-glycoprotein is elevated in many tumor cell lines and surgical specimens. Transfer and expression of wild-type *p53* genes may be able to modulate MDR1-mediated drug resistance, thus increasing the efficacy of chemotherapy.

With respect to transcriptional activation, a recent study implicates GADD45 as an inducible DNA repair component of the *p53*-mediated response to DNA damage. *p53* induces the expression of GADD45, whose gene product associates with proliferating cell nuclear antigen (PCNA), a protein needed for DNA repair. GADD45 itself can stimulate excision repair *in vitro*, and may therefore play a role in the removal of damaged bases produced by agents such as cisplatin and *N,N'*-bis(2-chloroethyl)-*N*-nitroso-urea (carmustine, BCNU). *p53* also induces the expression of *p21*(*waf-1*, *Cip1*, *Sdi 1*). The *p21* gene product is an inhibitor of several cyclin-dependent kinases (cdk) required for cell cycle progression and is a potent cell cycle suppressor by itself. As with GADD45, p21 associates with PCNA, and might thereby also play a role in DNA repair. Another cell cycle regulatory protein, whose loss is a feature of some cancers and most cultured tumor cell lines, is p16. p16 appears to specifically inhibit cdk 4, required for progression into S phase. Although not regulated by *p53*, p16 might nevertheless be a component of the cellular response to damage.

Another gene whose expression appears to be regulated by *p53* is *bax*. The *bax* gene is a member of the *bcl2* gene family, and may be an important component in the regulation of apoptosis. In association with *bcl2*, *bax* forms a heterodimer that blocks the anti-apoptotic activity of *bcl2*. A model has been proposed in which the susceptibility of a cell to apoptosis is determined by the ratio of *bax* to *bcl2*. *bax*, forming homodimers with itself, can actually induce apoptosis and could be a mediator of the *p53*-regulated response to DNA damage. Cells which fail to undergo apoptosis in the presence of *p53* may have insufficient levels of *bax*. Further studies will be needed to understand how each of these genes contributes to the pathways of apoptosis so critical for therapy sensitivity.

In conclusion, the genetic abnormalities involved in tumor pathogenesis have been shown to contribute to the development of treatment resistance. The loss of functional apoptotic pathways appears integral to the process of tumor evolution and to the acquisition of treatment resistance. Advances in our understanding of the genes responsible for tumorigenesis and apoptosis will lead to novel applications of gene therapy to restore apoptosis in cancer cells. These novel therapies will enhance the effectiveness of traditional chemotherapy and radiation treatments and will exploit the natural cellular mechanisms which eliminate cancer cells.

See Also the Following Articles

bcl-2 AND B-CELL NEOPLASIA: DYSREGULATION OF PROGRAMMED CELL DEATH IN CANCER; DRUG RESISTANCE: OVERVIEW OF MECHANISMS; MULTIDRUG RESISTANCE; *p53* TUMOR SUPPRESSOR GENE: STRUCTURE AND FUNCTION.

Bibliography

Fujiwara, T., Grimm, E. A., Mukhopadhyay, T., *et al.* (1994). Induction of chemosensitivity in human lung cancer cells in vivo by adenovirus-mediated transfer of the wild-type p53 gene. Cancer Res **54,** 2287–2291.

Gjerset, R. A., Turla, S. T., Sobol, R. E., *et al.* (1995). Use of wild-type *p53* to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. *Mol. Carcinog.* **14,** 275–285.

Hickman, J. A. (1992). Apoptosis induced by anticancer drugs. *Cancer Metast. Rev.* **11,** 121–139.

Kastan, M. B., Onyekwere, O., Sidransky, D., *et al.* (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res* **51,** 6304–6311.

Korsmeyer, S. J. (1992). Bcl-2: A repressor of lymphocyte death. *Immunol. Today* **13,** 285–288.

Levine, A. J., (1993). The tumor suppressor genes. *Annu. Rev. Biochem.* **62,** 623–651.

Lotem, J., and Sachs, L. (1993). Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents. *Blood* **82,** 1092–1096.

Lowe, S. W., Bodis, S., McClatchey, A., *et al.* (1994). p53 status and the efficacy of cancer therapy in vivo. *Science* **266,** 807–810.

Lowe, S. W., Ruley, H. E., Jacks, T., *et al.* (1993). p53-mediated apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74,** 957–967.

Miyashita, T., Krajewski, S., Krajewska, M., *et al.* (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* **9,** 1799–1805.

Miyashita, T., and Reed, J. C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80,** 293–299.

Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980). Cell death: The significance of apoptosis. *Int. Rev. Cytol.* **68,** 251–306.

Vol. 10, 545–554, August 1999

Inhibition of the Jun Kinase Pathway Blocks DNA Repair, Enhances p53-mediated Apoptosis and Promotes Gene Amplification[1]

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Abstract

We have previously shown, by expression of a nonphosphorylatable dominant inhibitor mutant of c-Jun [cJun(S63A,S73A)], that activation of the NH_2-terminal Jun kinase/stress-activated protein kinase by genotoxic damage is required for DNA repair. Here, we examine the consequences of inhibition of DNA repair on p53-induced apoptosis in T98G cells, which are devoid of endogenous wild-type p53. Relative to parental or wild-type c-Jun-expressing control cells, mutant Jun-expressing T98G clones show similar growth rates and plating efficiencies. However, these cells are unable to repair DNA (PCR-stop assays) and exhibit up to an 80-fold increased methotrexate-induced colony formation due to amplification of the dihydrofolate reductase gene. Moreover, the mutant c-Jun clones exhibit increased apoptosis and elevated bax:bcl_2 ratios on expression of wild-type p53. These results indicate that inhibition of DNA repair leads to accumulation of DNA damage in tumor cells with unstable genomes and this, in turn, enhances p53-mediated apoptosis.

Introduction

Tumor progression involves the rapid accumulation of genetic alterations, some of which promote cell proliferation and enable cell survival in changing environments. Genomic instability, one of the unique features of cancer, may provide the driving force for progression by facilitating these rapid genetic alterations (see Ref. 1). Nevertheless, this instability generates strand breaks and other forms of DNA damage that could be deleterious to cell survival. There is, therefore, selective pressure on the cancer cell to modulate its DNA damage response to insure survival while accommodating this increased genetic instability.

One way in which a cancer cell may modulate its DNA damage response is loss of the tumor suppressor p53. p53 mediates apoptosis in response to DNA damage, possibly as a result of its ability to recognize and bind to damaged DNA, including DNA containing single-stranded ends (2) and DNA in abnormal structures known as insertion-deletion loops (3). Stabilization of p53 protein occurs after DNA damage in a process that involves DNA-PK[3]/ATM as a key mechanism (4, 5). Numerous studies correlate loss of p53 with increased genome instability (6–9), aneuploidy (10, 11), and tumor progression (12), suggesting that loss of p53 renders cells permissive for further genome destabilizing events that accompany and promote tumor progression, such as gene amplification and deletion. Restoration of p53 function in tumor cells that no longer express wild-type p53 restores the DNA damage recognition pathways and leads to G_1 arrest or apoptosis (see Ref. 13, review).

DNA damage also leads to activation of the JNK/stress-activated PK (14). JNK phosphorylates the c-Jun component of the AP-1 complex and related transcription complexes on serines 63 and 73 in the NH_2-terminal domain, thereby greatly activating transcriptional transactivation by AP-1 and related c-Jun-containing complexes, such as the c-Jun/ATF2 heterodimer. JNK activity is strongly induced in response to a variety of DNA damaging treatments, such as UV irradiation (15), cisplatin (15, 16), camptothecin (17), and etoposide (18). We have previously shown that activation of the JNK pathway that follows DNA damage is required for DNA repair, suggesting an essential role of JNK in regulating the DNA repair process (16). Phosphorylation of c-Jun is also induced by certain oncogenes (19) and is required for c-Jun plus Ha-*ras* cotransformation of rat embryo fibroblasts (19, 20). Complete loss of c-Jun in transgenic mouse embryo fibroblasts results in proliferation defects leading to prolonged passage through crisis and delay of spontaneous immortalization (21).

To more fully understand the role of the JNK pathway and c-Jun phosphorylation in cellular transformation, tumorigenesis, and DNA repair, we have recently selected T98G glioblastoma cells modified to express a mutant Jun that acts as a dominant-negative inhibitor of wild-type c-Jun downstream targets. T98G cells express only mutant p53 (22) and, unlike many other cell types, including normal lung epithelial cells (23), they express elevated, easily detectable levels of

Received 12/29/98; revised 4/30/99; accepted 6/24/99.
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[1] Supported in part by National Cancer Institute Grants CA69546 (to R. A. G.), CA63783-05 (to D. A. M.), and CA76173-01 (to D. A. M.); Grant DAMD17-96-1-6038 from the Department of Defense (to R. A. G.); a grant from Introgen Therapeutics, Inc. (Houston, TX; to R. A. G.); and Grant 3CB-0246 from the Breast Cancer Research Program of the University of California (to D. A. M.).
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[3] The abbreviations used are: PK, protein kinase; JNK, Jun kinase; AP, activator protein; ATF, activating transcription factor; DHFR, dihydrofolate reductase; EGF, epidermal growth factor; ES, embryonal stem; HPRT, hypoxanthine phosphoribosyltransferase.

JNK activity, which can be activated an additional 5–10-fold by treatment with the DNA cross-linking agent cisplatin (16). The Jun mutant construct used to modify T98G cells was originally derived by Binétruy *et al.* (19) and Smeal *et al.* (20) and has alanine substitutions at serine positions 63 and 73. Mutant Jun, therefore, cannot be phosphorylated by JNK. Expression of mutant Jun does not alter the basal or induced levels of JNK activity in these cells, indicating that mutant Jun has no direct effect on the JNK enzyme.[4] However, it does strongly inhibit transactivation of AP-1 reporter plasmids in rodent fibroblasts (19, 20) and T98G cells,[4] indicating that mutant Jun acts as a competitive inhibitor in the formation of an active AP-1 complex and, therefore, greatly impedes phosphorylation-dependent transactivation functions of c-Jun (19, 20). Furthermore, in A549 human lung carcinoma cells, in which the JNK pathway is known to be required for the EGF-stimulated cell growth (23), inhibition of the JNK pathway by the application of high affinity JNK oligonucleotides leads to inhibition of EGF-dependent growth in a manner indistinguishable from that caused by stable expression of mutant Jun (23). Thus, stable expression of mutant Jun seems to be a potent and specific inhibitor of phosphorylation-dependent effects of endogenous c-Jun that are usually promoted by the action of JNK.

T98G cells that express mutant Jun have a marked increase in sensitivity to the DNA damaging drug cisplatin and to UV radiation, and this increased sensitivity to DNA damage correlates with an inability to repair DNA (16). This suggests that phosphorylation of the wild-type c-Jun subunit of transcription factors, such as AP-1 and the c-Jun/ATF2 heterodimer, may contribute to DNA repair and survival after DNA damage through induction of DNA synthesis and repair genes such as topoisomerase I and DNA polymerase β, both of which have functional AP-1 and ATF2/cAMP-responsive element binding protein sites (which bind to c-Jun/ATF2) in their promoters (24–27). Phosphorylation of c-Jun may also contribute to cell survival during the crisis phase of tumorigenic transformation by promoting repair of DNA strand breaks generated by the mechanisms that destabilize the genome during tumor progression.

Restoration of p53 function in T98G glioblastoma cells by exposure to p53-adenovirus promotes low levels of apoptosis at gene transfer efficiencies of 50–80% (28). We have found that levels of apoptosis can be significantly increased in these cells when they are treated with p53 adenovirus in combination with DNA damaging agents, such as cisplatin and radiation. This is consistent with a model in which the level of DNA damage sustained by the cell is a strong determinant of p53-mediated apoptosis, as suggested by Chen *et al.* (29). In this study, we hypothesized that inhibition of DNA repair by expression of mutant Jun, would also enhance p53-mediated apoptosis. It is known that various forms of genetic instability characteristic of cancer cells, including gene amplification, gene deletion, and broken chromosomes are related in origin through the involvement of strand breaks (see Ref. 30, review). By blocking DNA repair, mutant Jun is predicted to promote elevated levels of strand breaks, which then serve as signals for p53-mediated apoptosis. The elevated level of strand breaks could also stimulate further gene amplification. In the studies reported here, we extend and confirm our earlier observations that mutant Jun expression leads to inhibition of DNA repair. Moreover, we show that mutant Jun expression predisposes cells to gene amplification as judged by the amplification of the *DHFR* gene. We further show that expression of mutant Jun greatly enhances p53-mediated apoptosis. These observations provide support for the hypothesis that inhibition of DNA repair in cancer cells with unstable genomes enhances sensitivity to DNA damaging chemotherapy and p53-dependent apoptosis.

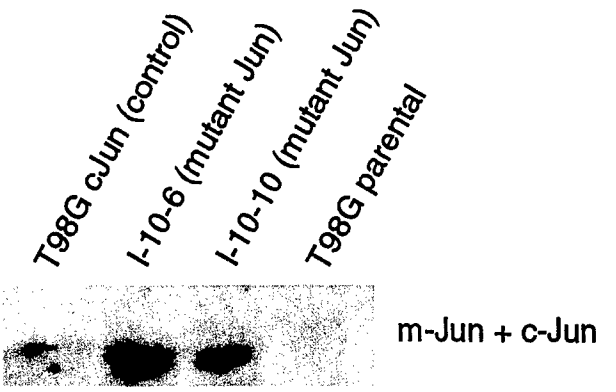

Fig. 1. Increased expression of immunoreactive Jun in stable transfectants. Western blot analysis of total c-Jun plus mutant Jun in lysates of control c-Jun-modified clone T98GcJun *(Lane 1)*, T98G mutant Jun-expressing clones I-10-6 *(Lane 2)* and I-10-10 *(Lane 3)*, and parental T98G cells *(Lane 4)*. Each lane represents the electrophoresis of 40 μg of total protein.

Results

T98G Mutant Jun-expressing Cells Resemble Parental T98G Cells with Respect to Growth Rate and Plating Efficiency. We have previously shown that mutant Jun-expressing T98G clone I-10-10 has decreased viability after treatment with cisplatin and other DNA-damaging agents, likely due to a defect in DNA repair (16). Here, we examine this and a second mutant Jun-expressing clone, I-10-6, as well as a control c-Jun-expressing clone (T98GcJun) and parental T98G cells for the expression levels of total immunoreactive Jun, for proliferation rates, and for plating efficiencies. Fig. 1 shows a Western blot of lysates from each of these four cell lines using an antibody that recognizes both mutant Jun and c-Jun. Equivalent loading was confirmed by stripping the blots and reprobing them with an anti-β-actin antibody (data not shown). As shown in the figure, mutant Jun-modified clones I-10-10 and I-10-6, as well as control c-Jun-modified clone T98GcJun, overexpress total Jun, consistent with expression of the exogenous constructs. The mutant Jun-expressing clones, as well as the control c-Jun-modified clone, show little difference from parental T98G cells with respect to proliferation rate or plating efficiency (Table 1). Only slight growth alterations were observed, but these did not correlate with expression of mutant Jun be-

[4] O. Potopova and D. Mercola, unpublished observations.

Table 1 Culture characteristics of T98G clones

Clone	Relative doubling time[a]	Plating efficiency[b]
T98G	1	47% ± 3%
T98GcJun (control)	1.0 ± 0.14	52% ± 17%
I-10-10	0.83 ± 0.10	42% ± 13%
I-10-6	1.2 ± 0.04	45% ± 2%

[a] Average of two experiments on different occasions, each in triplicate.
[b] Average of three experiments on different occasions, each in triplicate.

cause clone I-10-10 proliferates about 20% faster than parental cells or c-Jun-modified cells and clone I-10-6 proliferates about 20% slower. Therefore, expression of mutant Jun in T98G glioblastoma cells inhibits their ability to repair DNA damage (see below), but it is not growth suppressive in itself.

T98G Mutant Jun-expressing Cells Are Defective in Repair of Cisplatin Adducts. Cisplatin adduct formation and repair was analyzed by a PCR-based assay (PCR-stop assay), as described in "Materials and Methods." Because cisplatin adducts block PCR amplification by Taq polymerase, the intensity of the PCR signal derived from a given amplified region (in our case, the *HPRT* gene) is inversely proportional to the platination level and can be used as a quantitative measure of the number of cisplatin-DNA adducts on that region (31). Platination levels determined by PCR amplification of a given housekeeping gene have been shown to correspond to determinations of platination levels on genomic DNA by atomic absorption, demonstrating that the PCR method reflects global DNA platination levels (31). We have also observed in a variety of tumor cell lines that cells shown to be DNA repair deficient by the PCR assay were also defective in repairing a cisplatin-damaged reporter plasmid, as assayed by expression of the reporter gene 2 days after transfection.[5] The PCR assay was chosen for these studies because it measures repair of an endogenous genomic sequence. Fig. 2 shows the results of a PCR-stop assay performed on genomic DNA isolated from parental T98G cells, mutant Jun-expressing I-10-10 and I-10-6 cells, and control c-Jun-expressing T98GcJun cells after treatment with cisplatin, with and without a subsequent 16-h recovery period. The *bars* represent the relative amounts of PCR product resulting from PCR amplification of a 2.7-kb region of the *HPRT* gene of genomic DNA from cisplatin-treated *versus* untreated cells. In all cases, the results have been corrected for sample to sample fluctuations in PCR efficiency by normalizing the results to a 170-base PCR product of the same gene (*i.e.*, a fragment too small to register significant levels of platination and where fluctuation from sample to sample varied by <5%). The data show that treatment with 100 μM cisplatin results in an immediate decrease in the PCR signal intensity to about 85% of control signals obtained from DNA from untreated cells. On the basis of a Poisson relationship, this corresponds to an adduct density of about 0.16 adducts/2.7 kb. By 16 h after treatment, both T98G cells and c-Jun-expressing control cells (T98GcJun) have efficiently repaired the adducts, and the PCR signal strengths are equal to

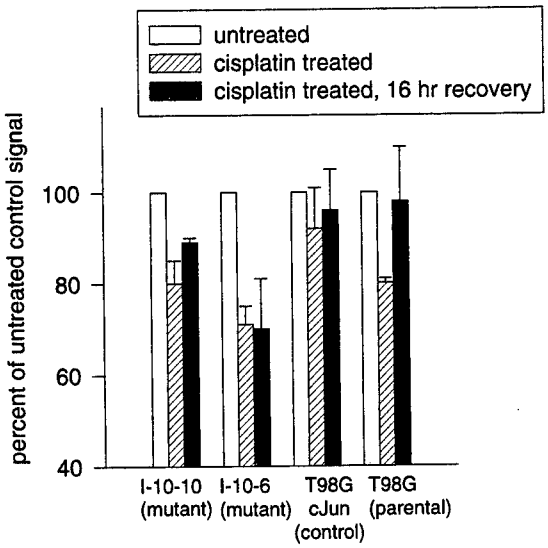

Fig. 2. PCR-stop assays of cisplatin adduct formation and repair. A 2.7-kb region of the *HPRT* gene was PCR amplified from genomic DNA from untreated cells or from cells treated 1 h ,15 min with 100 μM cisplatin and harvested either immediately or 16 h after treatment. *Bars*, the relative amounts of PCR product obtained from damaged *versus* undamaged templates. The results represent the averages of triplicate PCR reactions performed on two independent occasions.

controls (*i.e.*, no detectable adducts at 16 h). In contrast, mutant Jun-expressing clones I-10-10 and I-10-6 failed to repair the adducts, and PCR signal strengths remain unchanged after the 16-h recovery period (Fig. 2). These observations confirm and extend our earlier results (16) and strongly indicate that inhibition of the JNK pathway effectively blocks DNA repair.

T98G Mutant Jun-expressing Cells Give Rise to Methotrexate-resistant Clones with Higher Frequency Than Do Parental T98G Cells or c-Jun-expressing Cells. Certain types of DNA repair defects contribute to tumorigenesis (32, 33) and increased genome instability (34, 35). We examined the T98G clones described above for *DHFR* gene amplification, one measure of genome instability known to correlate with increased tumorigenicity after *in vivo* implantation of cells (36–38). T98G cells were plated in the presence of concentrations of methotrexate five times the LD_{50} and nine times the LD_{50} determined for these cells (*i.e.*, concentrations at which gene amplification of DHFR is known to be the predominant mechanism of resistance to the cytocidal effects of methotrexate; Refs. 39 and 40). Thus, the frequencies of appearance of methotrexate-resistant clones is a measure of genome instability. As shown in Table 2, T98G I-10-10 cells produce methotrexate-resistant colonies at about 20 times the frequency of the parental T98G cells, and T98G I-10-6 cells produce methotrexate-resistant colonies at about 80 times the frequency of parental T98G cells ($P = 0.006$). In contrast, stable expression of wild-type c-Jun had no effect on the frequency of resistance, indicating strongly that interference with a phosphorylation-dependent function of JNK predisposed cells to form resistant colonies.

To verify the occurrence of gene amplification in methotrexate-resistant colonies, several colonies were picked from

[5] R. A. Gjerset and A. Haghighi, unpublished results.

Table 2 Frequency of methotrexate-resistant colonies arising from T98G, T98GcJun, and T98G mutantJun clones I-10-10 and I-10-6

Cells were plated at a density of 10^5 cells/10-cm culture dish and incubated 4 weeks in the presence of methotrexate at the indicated concentrations. Plates were then stained with 70% methylene blue in methanol, and colonies were counted. For analysis of *DHFR* gene amplification, and colonies were counted. For analysis of *DHFR* gene amplification, several colonies were picked before staining and expanded, and cellular DNA was prepared and subjected to quantitative PCR analysis.

Clone	Methotrexate dose[a]	Frequency of colonies/10^5 cells (average of three experiments)	*DHFR* gene dosage relative to parental in representative colonies
T98G parental	5 × LD_{50}	2.3 ± 2.5	1
	9 × LD_{50}	0.3 ± 0.6	
T98GcJun (control)	5 × LD_{50}	2[b]	N/T
	9 × LD_{50}	0	
I-10-10	5 × LD_{50}	38 ± 19	3–4
	9 × LD_{50}	6 ± 5	
I-10-6	5 × LD_{50}	174 ± 21	2
	9 × LD_{50}	16 ± 12	

[a] LD_{50}, 0.1 μM (T98G and T98GcJun) or 0.04 μM (I-10-10 and I-10-6).
[b] One experiment.

each selection condition and expanded. Genomic DNA was prepared and subjected to quantitative PCR analysis using ^{32}P-labeled primers that define a 270-base fragment of the *DHFR* gene, which includes parts of exon 1 and intron A. PCR products were analyzed by agarose gel electrophoresis and quantitated by radioanalytic imaging, as described in "Material and Methods." The relative increase in PCR product from cellular DNA of methotrexate-resistant cells compared with unselected parental T98G cells was taken as a measure of the increased copy number of the *DHFR* gene and is indicated in Table 2. The methotrexate-resistant clones derived from T98G mutant Jun-expressing clones have from two to four times the gene dosage of the *DHFR* gene, relative to parental T98G cells, indicating that the observed methotrexate resistance reflected an increased *DHFR* gene copy number. We conclude, therefore, that inhibition of DNA repair by mutant Jun in T98G glioblastoma cells leads to accumulated DNA damage that can promote gene amplification.

T98G Mutant Jun Cells Are More Susceptible Than Are Parental Cells to p53-mediated Growth Suppression. T98G cells lack wild-type p53 function as a result of a methionine to isoleucine replacement in p53 at codon 237 (41). Restoration of wild-type p53 in T98G cells through gene transfer results in partial G_1 arrest (41) or apoptosis (28). Furthermore, agents that promote DNA strand breaks and other forms of DNA damage enhance p53-mediated apoptosis (28). On the basis of the observations above, indicating that mutant Jun-expressing cells are inhibited in DNA damage repair and predisposed to gene amplification, we predicted that strand breaks would accumulate in mutant Jun-expressing cells, thereby leading to increased p53-dependent growth inhibition and apoptosis. Fig. 3 compares the growth inhibition of Ad-p53-transduced cells relative to Ad-βgal-transduced cells 6 days after infection. The results represent the average of two experiments performed on

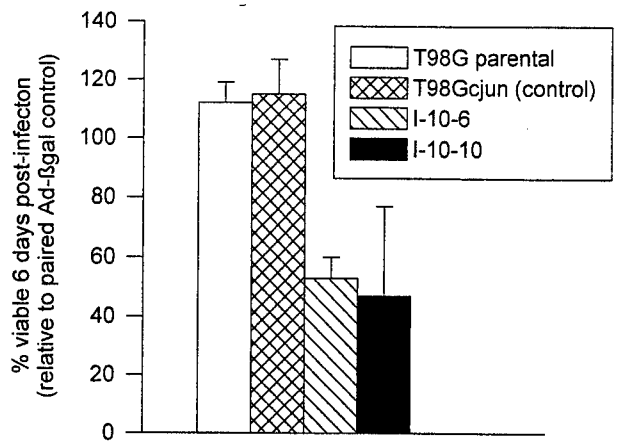

Fig. 3. Six-day viability assay of T98G subclones after treatment with Ad-p53, 100 pfu/cell for 3 h. Viability of Ad-p53-treated cultures is represented as a percentage of the same culture treated under identical conditions with Ad-βgal.

separate occasions, with each experiment being performed in triplicate. The infection efficiency, determined by X-gal staining of parallel cultures with Ad-βgal, was about 50% in all cases, low enough to cause incomplete growth suppression of parental T98G cells and control cells modified to stably express wild-type c-Jun, as shown in Fig. 3. Growth studies revealed that T98G I-10-10 and I-10-6 cells were considerably more growth suppressed upon expression of p53 under these conditions. Western blot analysis (Fig. 4) of the p53-responsive gene product *$p21^{waf1/cip1}$* in cell lysates 48 h after infection shows induction of $p21^{waf1/cip1}$ in all cases. The data, thus, show that $p21^{waf1/cip1}$ is not a crucial player in this setting. Equivalent loading was confirmed by stripping the blots and reprobing them with an anti-β-actin antibody (data not shown).

T98G Mutant Jun-expressing Cells Are More Susceptible to p53-mediated Apoptosis. To determine whether the p53-mediated growth inhibition of T98G mutant Jun-expressing cells observed in Fig. 3 could be accounted for by the induction of apoptosis, we assayed the cytoplasmic fractions of Ad-p53 or Ad-βgal-infected cells 48 h after infection for the presence of oligonucleosomal fragments (Fig. 5). These fragments are released from the nuclei of cells undergoing apoptosis and can be detected by an ELISA assay using antihistone antibodies and anti-DNA peroxidase antibodies. We assayed for apoptosis 48 h after exposure to p53 adenovirus or β-gal adenovirus because this is the point at which we have observed maximal transgene expression in Ad-β-gal-infected cells.[6] Fig. 5*A* shows the results of the ELISA assay on the various T98G cell clones. Low levels of oligonucleosomal fragment release similar to levels observed in uninfected cells were observed in Ad-β-gal-infected cells. Treatment of parental T98G cells and control wild-type c-Jun-expressing T98GcJun cells with Ad-p53 (100 pfu/cell, 3 h) resulted in virtually no induction of apoptosis under our

[6] Unpublished observations.

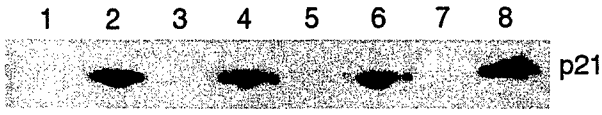

Fig. 4. Western blot analysis of $p21^{waf1}$ protein in lysates from T98G parental cells, mutant Jun-expressing clones I-10-10 and I-10-6, and control c-Jun-expressing clone T98GcJun 48 h after treatment with Ad-βgal or Ad-p53, 100 pfu/cell for 3 h. Each lane represents 40 μg of protein: *Lane 1*, T98G parental-Adβgal; *Lane 2*, T98G parental-Adp53; *Lane 3*, mutant Jun clone I-10-10-Adβgal; *Lane 4*, mutant Jun clone I-10-10-Adp53; *Lane 5*, mutant Jun clone I-10-6-Adβgal; *Lane 6*, mutant Jun clone I-10-6-Adp53; *Lane 7*, control T98GcJun-Ad-βgal; *Lane 8*, control T98GcJun-Adp53.

conditions, consistent with growth assays showing no suppression of overall growth after treatment of these cell lines with Ad-p53. However, readily detectable and significantly increased levels of apoptosis were observed in mutant Jun-expressing clones I-10-10 and I-10-6. These results are consistent with the appearance of Ad-p53-treated cultures as shown in Fig. 5*B*. Ad-p53-treated I-10-10 and I-10-6 cells lose contact with neighbors, become large, and contain cytoplasmic vacuoles. Thus, p53-mediated apoptosis is markedly enhanced in mutant Jun-expressing cells, possibly as a consequence of being triggered by endogenous strand breaks that fail to be repaired.

To confirm a p53-dependent mechanism of apoptosis, we carried out a Western blot analysis of the apoptosis regulatory proteins bax and bcl_2 in cells treated with Ad-p53 or Ad-βgal (Fig. 6). The levels of the proapoptotic effector bax, the gene of which is induced by p53 (42), increase after treatment with Ad-p53, as expected, whereas levels of the antiapoptotic protein bcl_2 remain largely unchanged. A comparison of the bax to bcl_2 protein is indicated by the ratios under the lanes in Fig. 6. The bax:bcl_2 ratio after treatment with Adp53 is significantly higher in mutant Jun-expressing cells I-10-10 and I-10-6 (ratios of 10 and 2.5, respectively) than in parental cells (ratio of 1.7) and c-Jun control cells (ratio of 0.8). Furthermore, a comparison of these ratios in uninduced *versus* induced cells (Adβgal-treated *versus* Adp53-treated) reveals a 3–4-fold increase for the Adp53-treated parental and cJun-expressing control cells compared with the same cells treated with Adβgal, whereas Adp53-treated mutant Jun-expressing cells I-10-10 and I-10-6 show an increase of some 8–25-fold, respectively, compared with the same cells treated with Adβgal. In accordance with other data suggesting that the bax:bcl_2 ratio is a critical determinant of apoptosis (see Ref. 43, review), these data support a role for bax in the increased apoptosis observed after Adp53 treatment of mutant Jun-expressing cells.

Discussion

In this study, we have examined how a dominant-negative inhibitor of phosphorylated wild-type c-Jun downstream targets affects cell proliferation, DNA repair, susceptibility to p53-mediated apoptosis, and *DHFR* gene amplification in T98G glioblastoma cells. The JNK/stress-activated PK pathway is a cellular DNA damage and stress-response pathway that is activated by a variety of signals, including mitogens such as EGF (23), oncogenes (19), and numerous DNA-damaging agents such as UV radiation and cisplatin (15–17). Phosphorylation of c-Jun by JNK activates the transcriptional potential of AP-1 and related transcription factors such as c-Jun/ATF2, which use c-Jun as a heterodimeric partner in the transcription complex. The nonphosphorylatable mutant Jun construct used in these and earlier studies resembles normal cellular c-Jun, except for two alanine replacements at positions 63 and 73. This change has been shown to abrogate the cotransformation properties of c-Jun with H-*ras* in rat embryo fibroblasts (19, 20), and to block EGF-induced proliferation of lung carcinoma cells (23). T98G glioblastoma cells expressing this mutant Jun have reduced viability after treatment with the DNA-damaging drug cisplatin (16), and this result is due to an inability to repair cisplatin adducts, as we show here and in an earlier study (16). Thus, the JNK pathway may promote cell survival during transformation and in response to DNA damage.

In this study, we extend the analysis of the T98G mutant Jun clones analyzed previously. We observe that they grow with similar doubling times and have similar plating efficiencies as parental T98G cells or c-Jun-modified control cells, indicating that stable expression of mutant Jun does not substantially alter DNA synthesis. However, methotrexate-resistant clones arising in the presence of $\geq 5 \times LD_{50}$ are generated at a 20–80-fold higher frequency in mutant Jun-expressing clones compared with parental T98G cells and the wild-type c-Jun-expressing clone T98GcJun. Under these conditions, resistance to methotrexate is known to be primarily due to amplification of the *DHFR* gene (39, 40). We confirmed a low, but detectable increase in *DHFR* gene copy number of about 2–4-fold compared with parental T98G cells by quantitative PCR analysis of genomic DNA isolated from several representative clones of methotrexate-selected T98G I-10-10 and I-10-6 cells. Although low, such an increase in copy number could explain the increase in methotrexate resistance observed in these cells and is supported by previous studies that showed that a low level of *DHFR* gene amplification was sufficient to confer resistance to methotrexate (44). Furthermore, mutant Jun-expressing T98G cells, which do not express endogenous wild-type p53, exhibited increased growth suppression and apoptosis after exposure to p53 adenovirus and restoration of wild-type p53 function. Therefore, mutant Jun alone had little effect on the growth properties of T98G cells but manifested a negative effect on growth in the presence of wild-type p53.

Our results demonstrate that expression of a nonphosphorylatable mutant Jun, but not c-Jun, leads to a defect in DNA repair and contributes to increased gene amplification, one manifestation of genomic instability in mammalian cells. These observations are consistent with other examples in which DNA repair defects are seen to underlie a genome instability phenotype (34, 35). The results suggest that the DNA repair defect associated with expression of mutant Jun may generate elevated levels of strand breaks in T98G cells compared with T98G parental cells and c-Jun-modified cells, both of which have an intact JNK pathway. The elevated level of breaks may, in turn, serve as initiation events for increased gene amplification (45), as well as triggers for DNA damage-

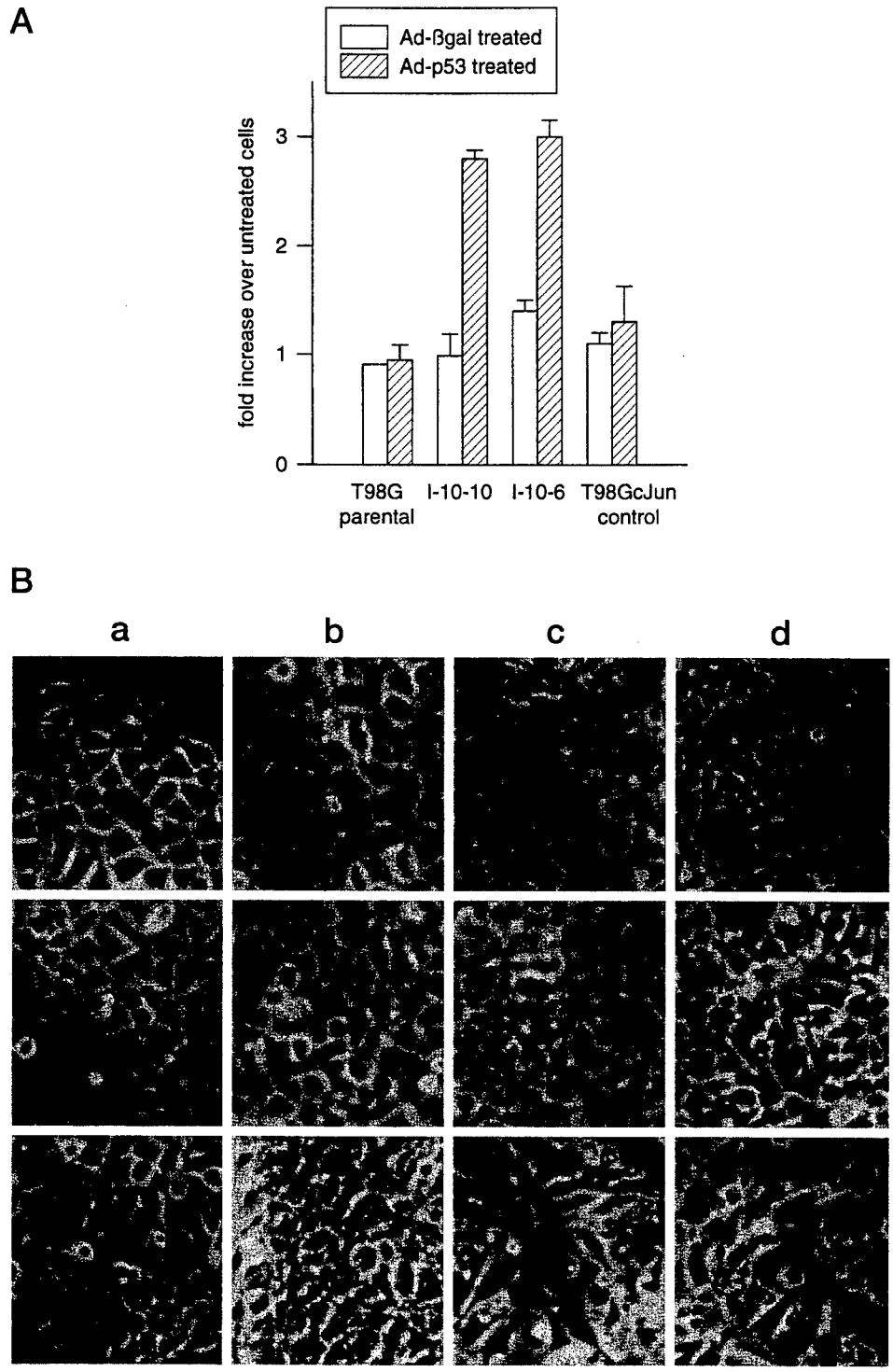

Fig. 5. *A*, ELISA apoptosis assay of cytoplasmic nucleosomes in untreated cells or in cells 48 h after being treated with 100 pfu/cell of Ad-βgal or Ad-p53 for 3 h. *B*, light microscopy (×40) of untreated cells (*top row*), or cells 72 h after treatment with Ad-βgal (*middle row*) or Ad-p53 (*bottom row*). *a*, T98G parental cells; *b*, c-Jun-modified clone T98GcJun; *c*, mutant Jun-modified clone I-10-6; *d*, mutant Jun-modified clone I-10-10.

induced stabilization of transduced wild-type p53, leading to apoptosis. Our results directly demonstrate both gene amplification and significantly increased p53-dependent apoptosis in mutant Jun-expressing cells in support of this hypothesis.

One possible explanation for our observations is that one or more downstream targets of wild-type c-Jun promotes repair of endogenous strand breaks. Candidate targets include DNA polymerase β, PCNA, topoisomerase I, topoisomerase II, and GADD153, all of which have potential AP-1 or c-Jun/ATF2 binding sequences in their promoter regions (see Refs. 24–27 and Ref. 46, review). In the cases of DNA polymerase β and topoisomerase I, these c-Jun/ATF2 binding sites are known to be functional and stress activated (46).

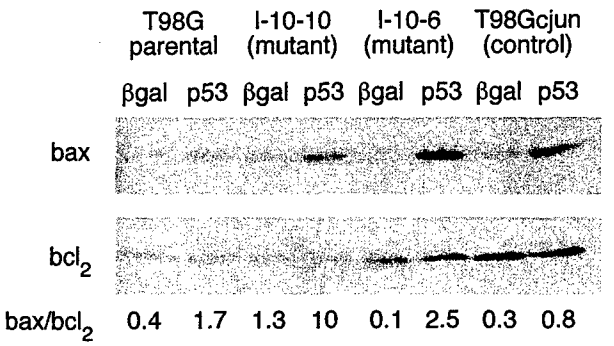

Fig. 6. Western blot analysis of bax and bcl_2 protein in lysates from T98G parental cells, mutant Jun-expressing clones I-10-10 and I-10-6, and control c-Jun-expressing clone T98GcJun 48 h after treatment with Ad-βgal or Adp53, 100 pfu/cell for 3 h. Each lane represents 15 μg of protein for the bax analysis and 30 μg of protein for the bcl_2 analysis. After immunostaining and band detection with enhanced chemiluminescence Western reagent, bands were quantitated using Kodak digital software. Ratios of bax to bcl_2 are indicated below the lanes. Experiment was carried out twice with similar results.

Moreover, all of these gene products have been implicated in the repair of cisplatin-DNA adducts (47). Thus, although an intact JNK pathway in T98G parental cells and in c-Jun-modified control cells would not directly prevent DNA damage-induced p53 stabilization, the pathway would act indirectly to attenuate p53-mediated apoptosis by efficiently promoting repair of endogenous strand breaks that would trigger p53 stabilization.

An additional mechanism also may play a role in cells expressing endogenous wild-type p53. Shreiber *et al.* (21) have recently shown that c-Jun directly down-regulates p53 expression through binding to a variant AP-1 site in the endogenous cellular p53 promoter. In their study, negative regulation of p53 by c-Jun seemed to be crucial to cellular transformation in that transgenic mouse embryo fibroblasts lacking c-Jun displayed proliferation defects, elevated p53 expression, and prolonged transit through crisis before spontaneous immortalization. Thus, c-Jun may attenuate p53-mediated apoptosis both by down-regulating expression of p53 and by promoting repair of endogenous DNA damage that could trigger p53 stabilization and apoptosis.

Two independently derived mutant Jun-expressing clones show similar properties, whereas a third clone expressing wild-type c-Jun and maintained in culture for a similar period did not share any of these properties. These observations strengthen the argument that down-regulation of DNA repair as a consequence of mutant Jun expression underlies the elevation in *DHFR* gene amplification and enhanced predisposition to p53-mediated apoptosis. Our results suggest, in addition, that increased expression of the p53-regulated pro-apoptotic effector bax leads to an increased bax:bcl_2 ratio that contributes to enhanced apoptosis in mutant Jun-expressing cells after exposure to p53 adenovirus. This is consistent with a variety of observations in other systems showing the importance of the bax:bcl_2 ratio in determining apoptosis (see Ref. 43, review). Thus, an elevated level of endogenous DNA strand breaks in mutant Jun-expressing cells may result in increased stabilization and activation of p53 and increased induction of bax.

The recent identification of p53 as a physiological substrate for JNK (48) indicates that the JNK response extends to other targets besides c-Jun, and these could mediate the various aspects of the stress response. Although inhibited in c-Jun phosphorylation, T98G cells modified with mutant Jun express constitutively active JNK at levels similar to the parental T98G cells.[7] They would, therefore, be expected to carry out phosphorylation of other JNK substrates similarly to parental cells. The ability of T98G mutant Jun cells to carry out apoptosis after restoration of p53 activity suggests that any JNK-related apoptotic functions are not disrupted by the mutant Jun modification.

Consistent with our observations that the mutant Jun modification has no significant effect on cell growth or plating efficiency of T98G cells is a study demonstrating that ES cells lacking c-Jun had similar viability and growth rate as parental ES cells and were able to efficiently transactivate AP-1 reporter constructs (49). Thus, most of the functions of c-Jun in ES cells seemed to be complemented by other Jun proteins. In our case, mutant Jun itself may be able to carry out the c-Jun functions required for basal growth. However, phosphorylation of c-Jun seems to be critical in the cellular response to DNA damage.

Our results can be understood in light of a growing body of evidence supporting a role for p53 in modulating apoptosis in response to DNA damage (see review, Ref. 50) and in proportion to the extent of damage (29). p53 is a DNA damage recognition protein known to bind to a variety of types of DNA damage, including single-strand ends (2), and insertion-deletion loops (3). These types of damage, which could serve as triggers for p53-mediated apoptosis, are likely to be generated in tumor cells by the mechanisms that promote spontaneous gene rearrangements, deletions, and amplifications. As such, a failure of DNA repair in mutant Jun-expressing cells would promote the accumulation of strand breaks, which would, on the one hand, favor gene amplification and other manifestations of genome instability and, on the other hand, promote DNA damage-induced stabilization of p53 and apoptosis.

As depicted in the scheme in Fig. 7, we hypothesize that activation of JNK and loss of p53 represent independent mechanisms by which tumor cells undergoing progression accommodate increased levels of genomic instability and insure survival while sustaining potentially lethal genome destabilizing events. By promoting DNA repair, the JNK pathway may limit damage to levels compatible with survival. Loss of p53 would further enhance survival owing to a down-regulated apoptotic response to unrepaired damage.

Materials and Methods

Cell Lines. T98G glioblastoma cells were obtained from Dr. Hoi U (University of California, San Diego, CA) and cultured at 37°C in 10% CO_2 in DMEM supplemented with 10% newborn calf serum. The T98G clones that had been modified to express mutant Jun, termed T98G-dnJun-I-10-10 and I-10-6 (see Ref. 16), or simply T98G I-10-10 and T98G I-10-6, were cultured in the same way as were T98G cells, except that 100 μg/ml hygromycin was added to the culture medium. The pLHC*mjun* vector

[7] O. Potopova, unpublished observations.

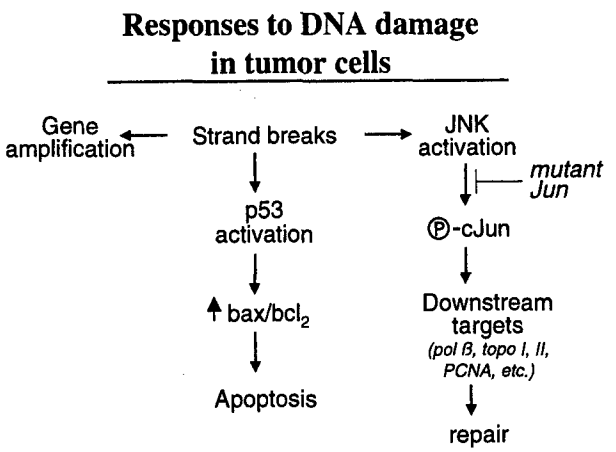

Fig. 7. Model explaining how, through inhibition of potential c-Jun downstream targets leading to DNA repair (*e.g.*, DNA polymerase β, topoisomerase I, II, PCNA), mutant Jun promotes the accumulation of endogenous DNA strand breaks in genomically unstable tumor cells and, thus, collaborates with p53 to promote p53-mediated induction of bax and apoptosis.

encodes a dominant negative mutant of c-Jun and was prepared, as described previously (51), by insertion of DNA encoding mutant Jun [obtained by site-directed mutagenesis by Smeal *et al.* (20)] into the retroviral vector pLHCX. Mutant Jun has ser → ala substitutions at positions 63 and 73, two sites of DNA damage-induced phosphorylation in wild-type c-Jun, and cannot be phosphorylated at these sites. As a control, T98G cells modified to overexpress wild-type c-Jun (T98GcJun) were obtained by cotransfection with a c-*jun* expression vector, pSV2c*jun* and with pSV2neo, and were cultured similarly, with the addition of 100 μg/ml G418.

Western Blot Analysis. Levels of total cellular Jun protein (c-Jun + mutant Jun), as well as levels of the gene products of the p53-regulated genes $p21^{waf1}$, *bax*, and bcl_2 were determined by Western blot analysis. Cell lysates (20–40 μg) were electrophoresed on a 12% acrylamide gel and blotted onto nylon membranes. Membranes were then treated with rabbit polyclonal anti c-Jun (1:200), or with mouse monoclonal anti $p21^{waf1}$ (1:200), or with rabbit polyclonal anti-bax (1:200), or with mouse monoclonal anti-bcl_2 (1:100), followed by an appropriate antirabbit or antimouse secondary antibody conjugated with horseradish peroxidase. All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used according to the protocol recommended by the manufacturer. Antibody reactive bands were revealed using the enhanced chemiluminescence Western detection system (Amersham Life Sciences, United Kingdom). For quantitation of bands, we used Kodak digital camera and analysis software.

Analysis of Repair of Cisplatin-DNA adducts. Cisplatin (*cis*-diamminedichloroplatinum) adduct formation and repair was analyzed by a PCR-based DNA damage assay (PCR-stop assay; Ref. 31). The assay is based on observations that Taq polymerase is blocked at cisplatin adducts, was used to analyze cisplatin adduct formation and repair. Because DNA fragments are platinated randomly, the distribution of damage fits a Poisson distribution, where a mean level of one adduct/fragment (*i.e.*, the portion of the genome defined by the forward and reverse PCR primers) will leave 37% of the fragments undamaged and these will be amplified to produce a PCR signal 37% of that from control DNA. For cisplatin treatments, cells were plated at 50% confluency in three wells of a 6-well plates in standard medium described above. After attachment, duplicate wells were treated with 100 μM cisplatin (Platinol, aqueous solution at 1 mg/ml, purchased from local pharmacies) for one h, 15 min, and one well was left untreated. After treatment, the untreated cells and one well of 100 μM cisplatin-treated cells were harvested, and genomic DNA was prepared. The remaining treated well was incubated an additional 16 h in the absence of cisplatin before harvesting. DNA was prepared using the QIAmp blood kit essentially following the manufacturer's protocol, except that cells were lysed directly on the plate in the presence of PBS, Qiagen protease, and lysis buffer supplied in the kit. After purification, DNA was adjusted to 0.5 mg/ml in sterile water and stored at −20°C. Quantitative PCR was used to compare cisplatin adduct formation on a 2.7-kb region of the *HPRT* gene. As an internal control for PCR efficiency, we PCR-amplified from the same templates a 170-base nonoverlapping region of the same gene. The smaller region represents a target too small to register significant levels of damage under our conditions. We found that both the 2.7-kb and 170-base products increased linearly with input template over the range 0.1–0.5 μg DNA/25 μl reaction and we, therefore, routinely used 0.125–0.25 μg template/reaction. Reactions were performed in 25 μl using 0.125–0.25 μg DNA, 25 pmol each of forward and reverse primer, 250 μM dNTPs (Pharmacia), 1.25 units of Taq polymerase (Qiagen), 1 × buffer (Qiagen), and solution Q (Qiagen). Bands were quantitated using a Kodak digital camera and analysis software. The amplification program was as follows: 1 cycle (94°C, 1 min, 30 s); 25 cycles (94°C, 1 min; 57°C, 1 min; 70°C, 2 min, 30 s); 1 cycle (94°C, 1 min; 57°C, 1 min; 70°C, 7 min). All assays were performed in triplicate on two separate occasions.

Virus. Replication-defective adenoviruses (Ad-p53 and Ad-βgal), in which the human p53 coding sequence or the bacterial β-galactosidase gene, respectively, replaced the viral early region *E1A* and *E1B* genes, were provided by Introgen Therapeutics, Inc. (Houston, TX).

Virus Treatments. Cells at 80% confluence were placed in DMEM supplemented with 2% heat-inactivated fetal bovine serum and infected for 3 h at a multiplicity of 100 pfu/cell. The efficiency of infection was determined by X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining a sample of the β-gal virus-infected cells (see Ref. 28) and was usually ≥50%.

Viability and Growth Assays. After infection, triplicate aliquots of cells were replated in 96-well plates at a density of 1000 cells/well. Plates were incubated for 5–7 days, and surviving cells were determined by adding a solution containing MTS [3-(4,5′-dimethylthiazol-2-yl)-5-(3-carboxymethoxylphenyl-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt] and PMS (phenazine methosulfate; both purchased from Promega, Madison, WI.) for 1 h and determining A590 nm of the resulting formazan product, following procedures provided by the manufacturer. For growth assays, cells were plated at 1000/per well in 96-well plates. On successive days from day 1 through day 8, triplicate samples were stained with MTS, as described above.

Generation of Methotrexate-resistant Clones. LD_{50} values for methotrexate were determined for the cell lines to be tested. Cells were seeded at a starting density of 10^3 cells/cm^2 and allowed to attach for 16 h. Methotrexate (Sigma Chemical Co., St. Louis, MO) was then added to a concentration of 5 × LD_{50} or 9 × LD_{50}, concentrations known to select for *DHFR* gene amplification (39, 40). Medium with fresh methotrexate was replaced weekly. When colonies developed and reached a size of about 100–200 cells (about 5 weeks), plates were washed in PBS and stained with 1% methylene blue in 70% methanol.

Analysis of *DHFR* Gene Copy Number. To verify *DHFR* gene amplification after selection in methotrexate, as described above, several clones were picked and expanded. Genomic DNA from these clones, as well as from parental unselected cells was prepared from about 10^6 cells in each case using the QIAamp Blood Kitô (Qiagen, Inc., Chatsworth, CA) and resuspended at 0.5 mg/ml in sterile H_2O. Quantitative PCR was performed in 50-μl aliquots containing 0.2 μg of DNA, 50 pmol each of forward and reverse primers defining a 270-bp region of exon 1 and intron A of the *DHFR* gene (see below), 50 mm of KCL, 10 mm of Tris (pH 8.3), 1.5 mm of $MgCl_2$, 250 mM dNTPs, 0.5 μl of Tac polymerase (Qiagen, Inc.), 10 μl of Q buffer (Qiagen, Inc.), and 1 pmol of radioactively end-labeled reverse primer (labeled with γ-^{32}P-dATP). PCR conditions were as follows: 1 cycle, 94°C (1 min, 30 s); 25 cycles, 94°C (1 min), 57°C (1 min), and 70°C (2 min, 30 s); 1 cycle, 94°C (1 min), 57°C (1 min), and 70°C (7 min). After PCR, 10 μl aliquots were electrophoresed on a 1% agarose gel. The gel was vacuum-dried for 2 h onto filter paper, and the PCR-amplified 270-bp band was quantitated using an Ambis4000 Radioanalytic Imaging system (Ambis, Inc., San Diego, CA). Quantitative conditions were established by demonstrating in control reactions with known amounts of DNA in 2-fold dilutions that product formation was directly proportional to input template. Primer sequences for the *DHFR* gene were: forward primer, 5′-GGTTCGCTAAACTGCATCGTCGC-3′, and reverse primer, 5′-CAGAAATCAGCAACTGGGCCTCC-3′. An increase in *DHFR* gene copy number was

then equal to the fold increase in the PCR product from cellular DNA of methotrexate-resistant clones compared with that of unselected parental cells.

Apoptosis Assay. Apoptosis was assayed using the Cell Death Detection ELISA (Boehringer Mannheim, Indianopolis, IN), a quantitative photometric peroxidase immunoassay that detects cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) that are released from the nuclei of cells undergoing apoptosis. Cells (2×10^5) were plated in 24-well plates and infected the next day (when the cells were about 80% confluent) with Ad-p53 or Ad-βgal, as described above. Forty-eight h after infection, cells were collected and cytoplasmic fractions were prepared and assayed for the presence of mono- and oligonucleosomes by following the manufacturer's protocol.

Acknowledgments

We thank M. Karin for providing the mutant Jun expression vectors and Dr. Hoi U for providing the T98G cells.

References

1. Nowell, P. C. The clonal evolution of tumor cell populations. Science (Washington DC), *194:* 23–28, 1976.

2. Bakalkin, G., Selivanova, G., Yakovleva, T., Kiseleva, E., Kashuba, E., Magnusson, K. P., Szekely, L., Klein, G., Terenus, L., and Wiman, K. G. p53 binds single stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain. Nucleic Acids Res., *23:* 362–369, 1995.

3. Lee, S., Elenbas, B., Levine, A., and Griffith, J. p53 and its 14-kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. Cell, *81:* 1013–1021, 1995.

4. Shieh, S-Y., Ikeda, M., Taya, Y., and Prives, C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell, *91:* 325–334, 1997.

5. Woo, R. A., McLure, K. G., Lees-Miller, S. P., Rancourt, D. E., and Lee, P. W. DNA-dependent protein kinase acts upstream of p53 in response to DNA damage. Nature (Lond.), *394:* 700–704.

6. Tainsky, M. A., Bischoff, F. Z., and Strong, L. C. Genomic instability due to germline p53 mutations drives preneoplastic progression toward cancer in human cells. Cancer Metastasis Rev., *14:* 43–48, 1995.

7. Agapova, L. S., Ilyinskaya, G. V., Turovets, N. A., Ivanov, A. V., Chumakov, P. M., and Kopnin, B. P. Chromosome changes caused by alterations of p53 expression. Mutat. Res., *354:* 129–138, 1996.

8. Bertrand, P., Rouillard, D., Boulet, A., Levalois, C., Soussi, T., and Lopez, B. S. Increase of spontaneous intrachromosomal homologous recombination in mammalian cells expressing a mutant p53 protein. Oncogene, *14:* 1117–1122, 1997.

9. Bouffler, S. D., Kemp, C. J., Balmain, A., and Cox, R. Spontaneous and ionizing radiation-induced chromosomal abnormalities in p53-deficient mice. Cancer Res., *55:* 3883–3889, 1995.

10. Donehower, L. A, Godley, L. A., Aldaz, C. M., Pyle, R., Shi, Y-P., Pinkel, D., Gray, J., Bradley, A., Medina, D., and Varmus, H. E. Deficiency of p53 accelerates mammary tumorigenesis in wnt-1 transgenic mice and promotes chromosomal instability. Genes Dev., *9:* 882–895, 1995.

11. Lanza, G., Jr., Maestri, I., Dubini, A., Gafa, R., Santini, A., Ferretti, S., and Cavazzini, L. p53 expression in colorectal cancer: relation to tumor type, DNA ploidy pattern and short-term survival. Am. J. Clin. Pathol., *105:* 604–612, 1996.

12. Gryfe, R., Swallow, C., Bapat, B., Redston, M., Gallinger, S., and Couture, J. Molecular biology of colorectal cancer. Curr. Probl. Cancer, *21:* 233–300, 1997.

13. Levine, A. J. p53, the cellular gatekeeper for growth and division. Cell, *88:* 323–331, 1997.

14. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. JNK1: a protein kinase stimulated by UV light and Ha-*ras* that binds and phosphorylates the c-Jun activation domain. Cell, *76:* 1025–1037, 1994.

15. Adler, V., Fuchs, S. Y., Kim, J., Kraft, A., King, M. P., Pelling, J., and Ronai, Z. Jun-NH_2-terminal kinase activation mediated by UV-induced DNA lesions in melanoma and fibroblast cells. Cell Growth Differ., *6:* 1437–1446, 1995.

16. Potapova, O., Haghighi, A., Bost, F., Liu, C., Birrer, M. J., Gjerset, R., and Mercola, D. The JNK/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. J. Biol. Chem., *272:* 14041–14044, 1997.

17. Saleem, A., Datta, R., Yuan, Z. M., Kharbanda, S., and Kufe, D. Involvement of stress-activated protein kinase in the cellular response to 1-β-D-arabinofunanosylcytosine and other DNA damaging agents. Cell Growth Differ., *6:* 1651–1658, 1995.

18. Osborne, M. T., and Chambers, T. C. Role of stress-activated/c-Jun NH_2-terminal protein kinase pathway in the cellular response to adriamycin and other chemotherapeutic drugs. J. Biol. Chem., *271:* 30950–30955, 1996.

19. Binétruy, B., Smeal, T., and Karin, M. Ha-*ras* augments c-Jun activity and stimulates phosphorylation of its activation domain. Nature (Lond.), *351:* 122–127, 1991.

20. Smeal, T., Binétruy, B., Mercola, D. A., Birrer, M., and Karin, M. Oncogenic and transcriptional cooperation with Ha-*ras* requires phosphorylation of c-Jun on serines 63 and 73. Nature (Lond.), *354:* 494–496, 1991.

21. Schreiber, M., Kolbus, A., Piu, F., Szabowski, A., Mohle-Steinlein, U., Tian, J., Karin, M., Angel, P., and Wagner, E. F. Control of cell cycle progression by c-Jun is p53 dependent. Genes Dev., *13:* 607–619, 1999.

22. Takahashi, J. A., Fukumoto, M., Kozai, Y., Ito, N., Oda, Y., Kikuchi, H., and Hatanaka, M. Inhibition of cell growth and tumorigenesis of human glioblastoma cells by neutralizing antibody against human basic fibroblast growth factor. FEBS Lett., *288:* 65–71, 1991.

23. Bost, F., McKay, R., Dean, N., and Mercola, D. The Jun kinase/stress-activated protein kinase pathway is required for epidermal growth factor stimulation of growth of human A549 lung carcinoma cells. J. Biol. Chem., *272:* 33422–33429, 1997.

24. Srivastava, D. K., Rawson, T. Y., Showalter, S. D., and Wilson, S. H. Phorbol ester abrogates up-regulation of DNA polymerase β by DNA-alkylating agents in Chinese hamster ovary cell. J. Biol. Chem., *270:* 16402–16408, 1995.

25. Kedar, P. S., Widen, S. G., Englander, E. W., Fornace, A. J., Jr., and Wilson, S. H. The ATF/CREB transcription factor-binding site in the polymerase β promoter mediates the positive effect of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine on transcription. Proc. Natl. Acad. Sci. USA, *88:* 3729–3733, 1991.

26. Baumgartner, B., Heiland, S., Kunze, N., Richter, A., and Knippers, R. Conserved regulatory elements in the type I DNA topoisomerase gene promoters of mouse and man. Biochim. Biophys. Acta, *1218:* 123–127, 1994.

27. Heiland, S., Knippers, R., and Kunze, N. The promoter region of the human type-I-DNA-topoisomerase gene. Protein-binding sites and sequences involved in transcriptional regulation. Eur. J. Biochem., *217:* 813–822 1993.

28. Gjerset, R. A., Turla, S. T., Sobol, R. E., Scalise, J. J., Mercola, D., Collins, H., and Hopkins, P. J. Use of wild-type *p53* to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. Mol. Carcinog., *14:* 275–285, 1997.

29. Chen, X., Ko, L. J., Jayaraman, L., and Prives, C. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev., *10:* 2438–2451, 1996.

30. Stark, G. R. Regulation and mechanisms of mammalian gene amplification. Adv. Cancer Res., *61:* 87–113, 1993.

31. Jennerwein, M. M., and Eastman, A. A polymerase chain reaction-based method to detect cisplatin adducts in specific genes. Nucleic Acids Res., *19:* 6209–6214, 1991.

32. Robbins, J. H., and Burk, P. G. Relationship of DNA repair to carcinogenesis in xeroderma pigmentosa. Cancer Res., *33:* 929–935, 1973.

33. Nacht, M., Strasser, A., Chan, Y. R., Harris, A. W., Schlissel, M., Bronson, R. T., and Jacks, T. Mutations in the *p53* and *SCID* genes cooperate in tumorigenesis. Genes Dev., *10:* 2055–2066, 1996.

34. Melton, D., Ketchen, A. M., Nuñez, F., Bonatti-Abbondandolo, S., Abbondandolo, A., Squires, S., and Johnson, R. Cells from ERCC1-

deficient mice show increased genome instability and a reduced frequency of homologous recombination. J. Cell. Sci., *111:* 395–404, 1998.

35. Lengauer, C., Kinzler, K. W., and Vogelstein, B. Genetic instability in colorectal cancers. Nature (Lond.), *386:* 623–627, 1997.

36. Sager, R., Gadi, I. K., Stephens, L., and Grabowy, C. T. Gene amplification: an example of accelerated evolution in tumorigenic cells. Proc. Natl. Acad. Sci. USA, *82:* 7015–7019, 1985.

37. Otto, E., McCord, S., and Tlsty, T. D. Increased incidence of *CAD* gene amplification in tumorigenic rat lines as an indicator of genomic instability of neoplastic cells. J. Biol. Chem., *264:* 3390–3396, 1989.

38. Tlsty, T. D. Normal diploid human and rodent cells lack a detectable frequency of gene amplification. Proc. Natl. Acad. Sci. USA, *87:* 3132–3136, 1990.

39. Brown, P. C., Tlsty, T. D., and Schimke, R. T. Enhancement of methotrexate resistance and dihydrofolate reductase gene amplification by treatment of mouse 3T6 cells with hydroxyurea. Mol. Cell. Biol., *3:* 1097–1107, 1983.

40. Tlsty, T. D., Brown, P. C., and Schimke, R. T. UV-radiation facilitates methotrexate resistance and amplification of the dihydrofolate reductase gene in cultured 3T6 mouse cells. Mol. Cell. Biol., *4:* 1050–1056, 1984.

41. Mercer, W. E., Shields, M. T., Amin, M., Sauve, G. J., Appela, E., Roman, J. W., and Ullrich, S. J. Negative growth regulation in a glioblastoma tumor line that conditionally expresses human wild-type p53. Proc. Natl. Acad. Sci. USA, *87:* 6166–6170, 1990.

42. Miyashita, T., and Reed, J. C. Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. Cell, *80:* 293–299, 1995.

43. Basu, A., and Haldar, S. The relationship between Bcl_2, Bax and p53: consequences for cell cycle progression and cell death. Mol. Hum. Reprod., *4:* 1099–1109, 1998.

44. Windle, B., Draper, B. W., Yin, Y. X., O'Gorman, S., and Wahl, G. M. A central role for chromosome breakage in gene amplification, deletion formation, and amplicon integration. Genes Dev., *5:* 160–174, 1991.

45. Smith, K. A., Agarwal, M. L., Chernov, M. V., Chernova, O. B., Deguchi, Y., Ishizaka, Y., Patterson, T. E., Poupon, M. F., and Stark, G. R. Regulation and mechanisms of gene amplification. Philos. Trans. R. Soc. Lond. B. Biol. Sci., *347:* 49–56, 1995.

46. Gjerset, R. A., and Mercola, D. Sensitization of tumors to chemotherapy through gene therapy. *In:* N. Nagy (ed.), Cancer Gene Therapy: Past Achievements and Future Challenges. New York, London, and Moscow: Plenum Publishing Corporation, in press, 1999.

47. Zamble, D. B., and Lippard, S. J. Cisplatin, and DNA repair in cancer chemotherapy. Trends Biochem. Sci., *20:* 435–439, 1995.

48. Milne, D. M., Campbell, L. E., Campbell, D. G., and Meek, D. W. P53 is phosphorylated *in vitro* and *in vivo* by an ultraviolet radiation-induced protein kinase characteristic of the c-Jun kinase, JNK1. J. Biol. Chem., *270:* 5511–5518, 1995.

49. Hilberg, F., and Wagner, E. F. Embryonic stem (ES) cells lacking functional c-Jun: consequences for growth and differentiation, AP-1 activity and tumorigenicity. Oncogene *7:* 2371–2380, 1992.

50. Harris, C. C. Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies. J. Natl. Cancer Inst., *88:* 1442–1445, 1996.

51. Potapova, O., Fahkrai, H., Baird, S., and Mercola, D. Platelet-derived growth factor-B/v-*sis* confers a tumorigenic and metastatic phenotype to human T98G glioblastoma cells. Cancer Res., *56:* 280–286, 1996.

Job: STARKEY 774-6 (MiMB 97-6.0)
Chapter: 29/Mercola
Pub Date: 2000

Operator: RH
Date: 1/3/01
Revision: Straight to pgs 495–520

29

Gene Therapy Approaches to Sensitization of Human Prostate Carcinoma to Cisplatin by Adenoviral Expression of p53 and by Antisense Jun Kinase Oligonucleotide Methods

Ruth Gjerset, Ali Haghighi, Svetlana Lebedeva, and Dan Mercola

1. Introduction

One of the challenges of medical research today is to find ways of bringing our genetic knowledge of cancer to clinical application and to develop improved therapies by exploiting gene-based strategies. By offering increased specificity and reduced toxicity, gene-based approaches promise alternatives when conventional treatments fail. Gene therapy also offers improved responses to conventional treatments when used in combination with gene therapy. Chief among the cancers in urgent need of improved treatment options is prostate cancer—the most commonly diagnosed cancer in men.

Advanced prostate cancer is resistant to most forms of hormone therapy, radiation therapy, and conventional chemotherapy. In fact, many of the most powerful chemotherapeutic drugs commonly used for other cancers are not effective against prostate cancer. Among these agents is cisplatin (also known as *cis*-diamminedichloroplatinum [CDDP], Platinol™, *cis*-platinum), which causes DNA damage in rapidly dividing cells by forming primarily bifunctional intrastrand adducts between adjacent guanines and guanine-adenine dinucleotides. Cisplatin is highly effective against ovarian cancer and bladder cancer and can achieve cures when used against testicular cancer. Cisplatin has also been applied to colon and brain tumors but is not in routine use for these applications *(1,2)*. A method for affecting increased sensitivity to cisplatin and for inhibiting or reversing resistance to cisplatin would provide an extended application for this drug, an important agent with well-known properties.

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From: *Methods in Molecular Biology, vol. 175: Genomics Protocols*
Edited by: M. P. Starkey and R. Elaswarapu

Job: STARKEY 774-6 (MiMB 97-6.0)
Chapter: 29/Mercola
Pub Date: 2000

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The cellular response to stress and DNA damage now appears to be central to the issue of resistance to DNA-damaging chemotherapies such as cisplatin, suggesting that genetic approaches that target these pathways may be effective in reversing resistance and enhancing the benefits of these agents. In this chapter, we describe the potential of reversing drug resistance through gene therapies that target two major stress and DNA damage-response pathways: the Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway and the DNA damage-induced apoptotic pathway mediated by the p53 tumor suppressor. There are suggestions that inhibition of the Jun kinase pathway or restoration of the p53 pathway may offer new biologic approaches to therapy sensitization in prostate cancer. These approaches would expand the potential of presently available treatments and offer alternatives when conventional treatment protocols fail. These approaches are illustrated by the use of a p53 adenoviral expression vector and by the use of highly efficient antisense JNK oligonucleotides.

Au: Is p53 a gene (therefore ital?)

1.1. In Vitro Combination Studies with p53 Adenovirus and Cisplatin

The p53 tumor suppressor has attracted considerable attention, not only for its tumor suppressor properties but also for its ability to sensitize tumor cells to apoptosis following treatment with chemotherapy and radiation *(3–8)*. Recent studies suggest that p53 gene therapy could have a broad application as a therapy sensitizer in cancer, a possibility that greatly expands the clinical application of p53-based approaches. Because many cancers, including prostate cancer, acquire p53 mutations as the disease progresses and becomes resistant to therapy, p53 gene therapy in combination with chemotherapy could have advantageous application for these advanced cancers. Numerous in vitro studies now support the application of p53 gene therapy as a therapy sensitizer for cancer *(3–8)*.

The results of screening tumor cells for sensitivity to various chemotherapeutic agents following exposure of cells to a replication-defective adenovirus encoding wild-type p53 (Adp53) compared to control vector (Ad-β-galactosidase [AD-βgal]) is summarized in **Fig. 1**. The cells were treated under conditions in which some 50–70% of them shows transgene expression, as evidenced by X-gal staining of parallel cultures infected with Ad-βgal. Viability is scored by the methanethiosulfonate (MTS) assay *(9)*. Under these conditions, it was found that growth suppression by Adp53 alone is incomplete and that 7-d viabilities of Adp53-treated cultures were about 60–80% of control vector–treated cultures. In addition, numerous tumor cell types lacking wild-type p53 expression, including the prostate cancer line PC-3, can be sen-

Fig 1

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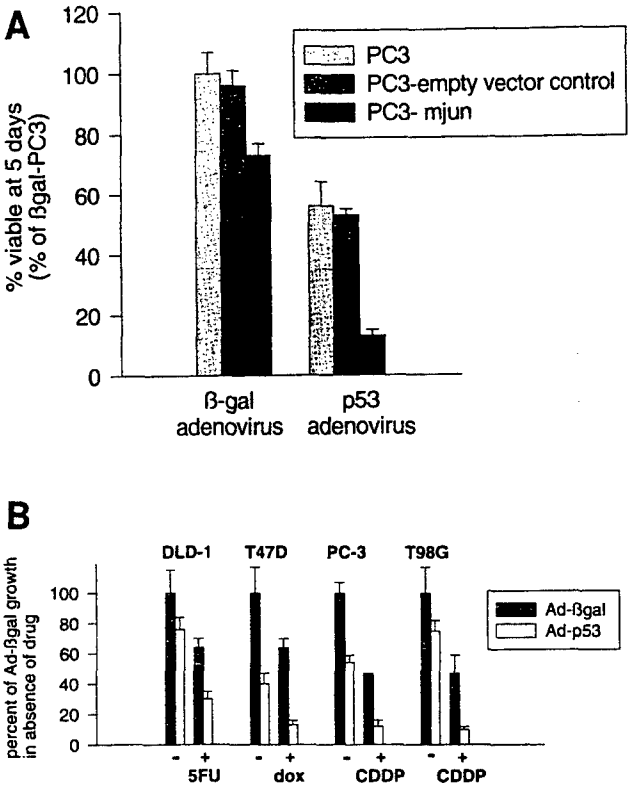

Fig. 1. **(A)** Effect of various chemotherapeutic agents on tumor cells. Ad53 is the wild type whereas Ad-βgal is the control vector. The viability was scored by MTS assay. **(B)** Sensitivities of mutant p53-expressing cell lines to Adp53 and chemotherapeutic drugs. Viability assay showing that Adp53 suppresses growth and enhances sensitivity to DNA-damaging chemotherapeutic drugs in p53 mutant–expressing cells (DLD-1 colon carcinoma cells, T47D breast cancer cells, PC-3 prostate cancer cells, and T98G glioblastoma cells). Infection efficiencies were 60–70%, and drug treatments 1 d postinfection were as follows: 5-fluorouracil (5FU), 10 μ*M* 1 h;, doxorubicin (dox) 3.7 μ*M* 1 h, cisplatin (CDDP), 30 μ*M* for 1 h for PC-3 cells and 20 ~*M* 1 h for T98G cells. Viability was assayed 6 d post drug treatment in all cases except PC-3, in which viability was assayed 4 d post drug treatment and expressed as a percentage of control cells treated with Ad-βgal.

sitized to chemotherapeutic drug treatment following restoration of wild-type p53 activity, consistent with the role of p53 in mediating DNA damage recognition and apoptosis.

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1.2. In Vivo Combination Studies Using p53 Adenovirus and Cisplatin in a Nude Mouse Model for Prostate Cancer

Recently it has been shown that p53 is highly effective at suppressing the growth of human prostate xenografts in nude mice but fails to achieve complete tumor eradication by itself *(10,11)*. We have tested the possibility that more complete suppression can be achieved by combining p53 gene therapy with the DNA-damaging chemotherapy, cisplatin. In the experiment shown in **Fig. 2**, with these treatment doses, both Adp53 and cisplatin administered as single agents led to a significant reduction in tumor growth relative to tumors receiving only control vector ($p < 0.001$). When combined, Adp53 and cisplatin led to an even greater reduction in tumor growth, which was significantly better than either agent used alone ($p < 0.02$). The vector doses in this experiment were low (equivalent to about 10–30 plaque-forming units [PFU]/cell) and derive completely without side effects, as judged by periodic weight measurements of the animals and histologic examination of tissues, suggesting that higher doses or more prolonged treatment could have been applied without adverse side effects. This in vivo study therefore confirms the in vitro observations and supports the clinical application of p53 adenovirus combination approaches to tumors expressing mutant p53.

Fig 2

Adenovirus-based approaches are among the most promising for clinical applications of gene therapy. Adenoviruses are relatively easy to prepare, are stable, can be obtained in high titer, and provide high gene-transfer efficiencies. Adenoviruses are also well tolerated in patients and have been used in phase I clinical trials with few side effects even with repeated doses *(12–18)*. Improvements in tissue targeting of adenovirus will extend the application of adenovirus-based approaches to a broad range of clinical situations. Nevertheless, adenovirus-mediated p53 gene transfer has already shown efficacy in clinical trials for lung cancer, hepatocellular carcinoma, and head and neck cancer *(18)*.

1.3. In Vitro Studies Combining Inhibition of the JNK Pathway with Chemotherapy

Another important cellular pathway involved in the DNA damage response is the JNK/SAPK pathway, which is often upregulated in prostate cancer *(19,20)*.

This pathway represents one of the mitogen-activated protein kinase pathways and plays a role in growth factor signaling, oncogene expression, and cellular transformation, as well as the DNA damage response *(9)*.

Numerous reports document the activation of the JNK pathway in cells following treatment with a variety of DNA-damaging agents, including ultravio-

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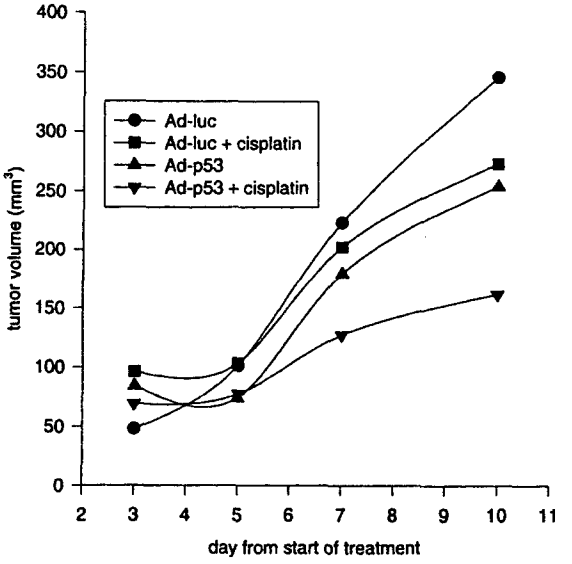

Fig. 2. Suppression of PC-3 prostate tumor growth in nude mice by Adp53 + cisplatin. Vector (Adp53 or control Ad-Luc, 10^8 PFU/tumor) was administered intratumorally on days 3, 5, 7, and 10, and cisplatin was administered intraperitoneal on d 1 and 8.

let (UV) radiation, ionizing radiation, methylethane sulfate, *N*-nitro-*N'*-nitroso-guanidine,1-β-D-arabinofuranosylcytosine), and cisplatin ***(19)***. A major substrate of JNK is the transcription factor c-Jun, which is greatly activated on phosphorylation of serine residuals 63 and 73. We have demonstrated that tumor cells that stably express a nonphosphorylatable dominant negative mutant cJun (c-Jun [863A, 573A]) are defective in repair of cisplatin adducts, consistent with their increased sensitivity to cisplatin (**Fig. 3**). The DNA repair assay that we used was based on the observations that cisplatin adducts will inhibit the *Taq* polymerase such that the decrease in yield of the polymerase chain reaction (PCR) product is directly proportional to the degree of platination of the template. The density of adducts can then be calculated based on a Poisson distribution predicted from a random process of platination ***(21)***. Thus, (*P*) the relative PCR signal strength (damaged template relative to undamaged template) is related to the platination level (*n*), the number of adducts per PCR amplicon-sized fragment by the formula $P = e^{-n}$. In our case, we amplified a 2.7-kb fragment of the HPRTase gene, a fragment providing a sufficiently large target size to enable us to detect significant decreases in PCR

Fig 3

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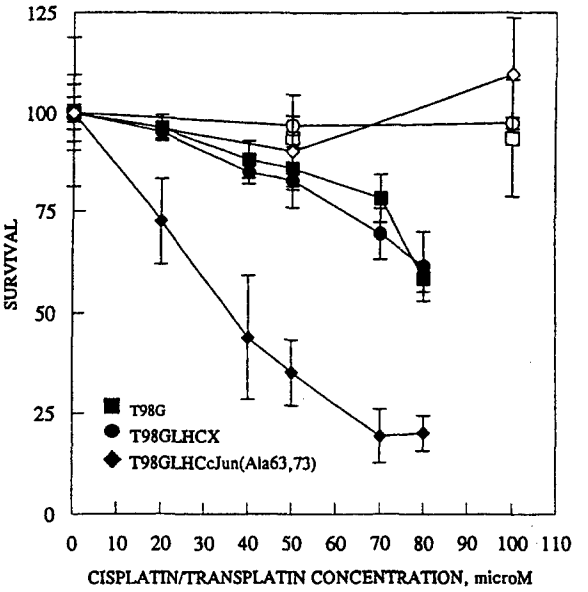

Fig. 3. Cisplatin and Transplatin sensitivity of T98G cells and clones. Cell survival (viability) assay showing that expression of mutant Jun (dnJun) sensitizes clonal T98G cells (■) to cisplatin. As controls, similar viability results are shown for wild-type c-dun overexpressing clonal T98G cells (◆), and empty vector expressing clonal T98G cells (●). Open symbols represent the corresponding experiments with the inactive transplatin control indicating that decreased survival by inhibition of the JNK pathway is specific to cisplatin-generated DNA damage (after **ref. *22***).

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signals from templates from cisplatin-treated cells. Amplification of a smaller fragment of 150 bp in length, a fragment too small to register significant levels of platination under our conditions, served as a control for the efficiency of the PCR amplification. **Table 1** summarizes the frequency of adduct formation calculated after quantitating the PCR signal strengths (results of two experiments performed in triplicate). As shown in **Table 1**, cisplatin adducts form at a greater frequency on genomic DNA in mutant Jun-expressing cells than in parental cells. In addition, mutant Jun-expressing cells show no repair of adducts in a 6-h recovery period following a 1-h treatment with cisplatin, whereas parental cells repair about half of the adducts in that time period.

Table 1

These results have been confirmed using a plasmid recovery DNA repair assay (**Fig. 4**). In this assay, cells are transfected with chloramphenicol acetyltransferase (CAT) reporter plasmids that have been damaged ex vivo by previous treatment with 25 μ*M* cisplatin for 3 h. Reporter gene expression 24

Fig 4

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Table 1
Cisplatin-DNA Adducts per 2.7 kb

Treatment	T98G Parental cells	T98G Mutant Jun cells
200 μ*M* cisplatin for 1 h	0.48 ± 0.08	0.63 ± 0.2
200 μ*M* cisplatin for 1 h, 6 h recovery	0.20 ± 0.05	0.55 ± 0.2

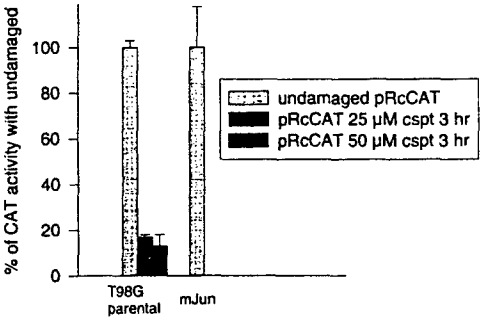

Fig. 4. Plasmid reactivation assay using CAT reporter plasmid damaged ex vivo by treatment with cisplatin (cspt). Reporter gene expression, assayed 24 h post-transfection, indicates that mutant Jun-expressing clonal T98G cells are suppressed in their ability to repair and express the CAT transgene.

and 48 h posttransfection is taken as an indication of DNA repair. As shown in **Fig. 4**, cells that express mutant Jun are suppressed relative to parental cells in their ability to repair the damaged plasmid, consistent with the PCR-based DNA repair assays.

Although the mechanism by which the JNK pathway affects DNA repair is not known, there are several candidate target genes whose roles in DNA synthesis and repair could account for observations that we have made. The promoters of DNA synthesis and repair genes contain AP-1 or ATF2/CREB regulatory elements (**Table 2**). These include DNA polymerase β, topoisomerase I, topoisomerase II, uracyl glycosylase, proliferating cell nuclear antigen, and metallothionine. In several cases, such as DNA polymerase β, exposure of cells to UV radiation is known to induce the gene, and ATF2/CREB sites are required for this induction. Thus, a number of genes involved in DNA excision repair may be coordinately upregulated on activation of the JNK pathway following cisplatin damage. Some or all of these could serve as potential targets for gene therapy approaches to drug sensitization of cancer.

Au: Define AP-1 & ATF2/CREB

Table 2

Table 2

DNA Repair Associated Genes Containing Potential JNK-Regulated Sequences (after ref. *61*)[a]

Repair Associated Gene	Element	Sequence (consensus sequence)2	LLG Position	Score	Reference for function
DNA polymerase B	AP-1/TRE	CTGACTCA (-tgactca)	337	2.0	It is known to be functional and TPA-activated classic TRE *(30)*.
	ATF/CREB	TTACGTAA (ttacgtca)	282	2.0	It is known to be genotoxic-activated *(30,*
DNA polymerase a *(32)*	ATF/CREB	CACGTCA (t/gacgtca)	−82		The function of ATF/CREB *(32)* is unknown.
		GGGGTCA (tgagtca)	−149		AP-1 sites are thought to be significant in DNA repair: DNA polymerase is over expressed in cisplatin-resistant cells and anti-Fos ribozyme sensitizes *(27)*.
Topoisomerase I	AP-1	GGGGGCGG (tgagtca)	753	2.0	*(33,34)*
	AP-1	TGACCCA (tgactca)	217	2.0	*(33,34)*
	ATF/CREB	TGACGTCA (tgacgtca)	792	2.0	It is known to be functional and stress activated (33, 34).
Topoisomerase IIα	ATF/CREB	TGACGCCG (tgacgtca)	286	2.0	*(35,36)*; Topo II is UV inducible and functions early in UV-induced DNA damage repair.
Topoisomerase IIβ	AP-1	TGATTGG (tgagtca)	337		*(35)*
	AP-1	TGACTCA (tgactca)	3		*(37)*

	AP-1	AGAGTCA (t g a g t c a)	65		*(37)*
ATF-3	AP-1	TGAGTAA (t g a c g t c a)	−1600		*(38,39)*; ATF-3 is stress-induced, anisomycin (JNK activator)-induced, and induced by ATF-2/c-Jun coexpression suggesting a functional role for the ATF/CREB site. ATF-3/c Jun heterodimers bind ATF/CREB sites and activate transcription *(40,41)* and ATF-3/c-Jun and ATF-3/JunD heterodimers have been shown to bind TTAGTTAC, a ATF/CREB sequence, which mediates EGF/ras/raf-stimulated transcription *(42)*, however, a role in induction of DNA repair genes is not known.
	AP-1	ATAGTCA (t g a c g t c a)	−1353		
	AP-1	AGACTAA (t g a c g t c a)	−605		
	AP-1	GAGTCA (t g a c g t c a)	−380		
	ATF/CRE	TTACGTCA (t t a c g t c a)	−92		
c-Jun	ATF/CREB	TTACCTCA (t t a c g c a)		2.0	*(43–45)*; The "functional" association with DNA repair is strong induction of c-Jun by genotoxins known to activate JNK/SAPK.
Uracil glycosylase *(46)*	AP-1	TGGGTCA (t g a g t c a)	141	2.0	Activation regulation is not known.
PCNA	AP-1	TGACTCA (t g a c t c a)	489	2.0	DNA polymerase-A accessory protein function.
PCNA	ATF/CREB	TGAGGTCAGGG (t g a c g t c a - - -)	209	1.64	*(47,48)*; 11-2, a potent JNK/SAPK activator, induces PNCA expression via ATF/CREB promoter sites, which is blocked by rapamycin.
	ATF/CREB	GTGACGTCAC (- t t a c g t c a - -)	1253	1.60	
GADD153 *(49)*	ATF/CREB	ACTCCTGACCTT (t g/t a c g t c a - - - -)	207	1.63	Induction requires phosphorylation-dependent event that is not PKA, PKC *(50)*, or p38 *(51)* mediated consistent

(continued)

Table 2 (continued)

Repair Associated Gene	Element	Sequence (consensus sequence)2	LLG Position	Score	Reference for function
Growth arrest and DNA-damage-inducible gene	AP-1	TGACTCA (t g a c t c a)	710	2.0	with a role for JNK/SAPK *(50)*. Moreover GADD153 is induced by MMS *(50)* and cisplatin *(52,53)*. The role of the ATF/CREB site is unknown. GADD153isphoshorylated and activated by p38 in response tostress *(53)*.
XRCC1 *(54,55)*	ATF/CREB	ACGTCA (a c g t c a)	1815	2.0	The site at 466 is consistent with c-Jun/ATF-3 vs. ATF-2 (TESS).
X-ray damage repair cross complementing gene product.	ATF/CREB	GGACGTCAA (t a c g t c a)	1814	2.0	Functional roles of these ATF/CREB and AP-1 sites are not known.
	ATF/CREB	CCTGACCTCA (- - t g a c g t c a)	2029	1.64	
	ATF/CREB	GCTGACGTCAG (- - t g a c g t c a -)	466	1.60	
		CCAATCA (t g/t a c/g t c a)	93	2.0	
MGMT *(56)*	ATF/CREB	TGCGTCA (t g a c g t c a)	1661	2.0	MGMT is induced by genotoxic agents *(57)*. The site at 1674 is consistent with c-Jun/ATF-3 (TESS). The functional significance of these sites is unknown.
06-Methylguanine-DNA-methyl-transferase	ATF/CREB	GTGACATCAT (- t g a c t c a -)	1195		
	AP-1	TGAGTCA (t g a g t c a)	734	2.0	
	AP-1	TTACTCA (t t a c t c a)	285	1.73	

MSH2 *(58,59)*	ATF/CREB	TGGCGTCA (t g a c t c a)	108	1.62	TESS does not recognize c-dun participation at 108 site. Role in cisplatin induced repair unknown.
	AP-1	TGAATCA (t g a c/g t c a)	569	2.0	MSH2 has been reported to selectively bind to cisplatin-DNA adducts *(32,58)*
	AP-1	TGATGAAA (t g a c/g t c a)	884	1.62	
MetallothioneinIIA	AP-1	GAGCCGCAAGT (g a g t c a - - - - t GACTTCTAGCG g a c t c a a g t c CGGGGCGTG a - - - - - - t g)	188	2.0	*(60)*; It is TPA and UV-light activated.

[1]Repair-associated protein for which only partial promoter sequences are known (i.e., in Genbank) without recognizable AP-1-regulated sites include ADP ribose polymerase, tif2/ref1, SSRP, Ercc1, and thymidylate synthetase.

[a]AP-1: activator protein-1 complex, a Jun and a Fos family member; ATF/CREB, cAMP: response element binding proteins; LLG: log likelyhood score, which is 2 for a perfect match of the candidate response, element with the consensus sequence (TESS criteria) and all ambiguous matches yielding a score of 0. MMS: methyl methanesulfonate; TESS: transcription element search system; PCNA, proliferating cell nuclear antigen; PKA, protein kinase A; PKC, protein kinase C.

[b]AP-1 consensus: TG/TAC/GTCA; CREB/ATF–2 consensus: TG/TACGTCA.

[c]Positions are based on TESS numbering of promoter sequences unless preceded by (–).

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1.4. In Vivo Studies Combining Inhibition of the Jun Kinase Pathway by Antisense Targeting JNK with Chemotherapy

In a parallel arm of the in vivo study shown in **Fig. 2**, we tested the antitumor efficacy of downregulation of JNK combined with cisplatin. In this study, we downregulated JNK with antisense oligonucleotides targeting either the JNK1 or JNK2 family of JNK isoforms. These compounds were previously developed and characterized as described *(22,23)*.

An important methodological consideration is the use of high affinity antisense oligonucleotides that completely suppress target mRNA and protein at low concentration. In collaborative studies with Isis Pharmaceuticals, we have developed a systematic method that now has been adapted to a multiwell procedure. The salient features are that a large series of phosphorothioate oligonucleotides complementary to 20 nucleotide stretches of JNK1 and JNK2 spaced approximately every 50 bp along the transcribed portion of the gene are prepared on a small scale. Thus, the target gene sequence must be known. These trial oligonucleotides are then tested for the ability to suppress steady-state transcript levels in culture cells 24 h after a 4-h lipid-mediated transfection *(22,23)*. Most of these trial oligonucleotides we tested were not efficient at promoting suppression of target mRNA at low (0.2–0.4 μM) concentration. However, it was readily possible to identify antisense compounds that were >90% efficient at eliminating target mRNA at these low concentrations after a single 4-h transfection. This step is key in avoiding any temptation to use less efficacious antisense compounds by increasing the concentration to high levels (>1 μM) at which many nonspecific interactions leading to aberrant cellular localization and weak membrane-protein complex formation have been observed *(24)*. Moreover, compounds that require >1 μM cannot be considered as potential drugs owing to the generation of nonspecific interactions and to the many drawbacks in attempting to achieve local concentrations on the order of ~1 μM in vivo. These considerations are likely important in understanding the many difficulties some researchers have had in "getting antisense to work."

For the studies summarized here, it was possible to select highly efficient antisense oligonucleotides that are complementary to a sequence that is invariant in all isoforms of JNK1 or JNK2, thereby making it possible to reduce or eliminate all isoforms of JNK1 or JNK2 with a single antisense oligonucleotide. Next, the elapsed time of suppression of target mRNA and target protein was determined *(23)*. These studies showed that a single antisense treatment suppressed target mRNA and protein from approximately 24 h to 72 h, thereby defining a 2-d window in which antisense-mediated effects could be observed *([22,23]*; unpublished data). However, for growth studies in which cell numbers are counted or tumor volume is measured, any loss of growth owing to

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inhibition of a growth-promoting target protein will remain apparent as decreased total growth for at least 3 wk *(22)*.

Because a variety of human tumor cells, including PC-3 cells, were sensitized to the cytotoxic effects of DNA-damaging cisplatin in vitro upon inhibition of the JNK pathway *(9)*, it was decided to test whether xenografts of PC-3 cells could be sensitized to cisplatin. Moreover, because many of the potential JNK-regulated genes encode gene products that facilitate DNA synthesis (**Table 2**), it appeared logical to expect that inhibition of JNK in vivo may affect tumor growth even in the absence of cisplatin *(25)*. This experiment was carried out as a separate arm of the same in vivo study as for the evaluation of Adp53 in **Fig. 2**.

Following inoculation of the mice with the PC-3 cells, the animals were monitored until visible and palpable tumors developed. At this point and for all subsequent observations, tumor volumes were estimated from the length and width of the tumors (*see* **Subheading 3.2.**). Treatment consisted of intraperitoneal injection of oligonucleotide solution (*see* **Subheading 2.2.**) daily for 6 of 7 d/wk. On the d 7, oligonucleotide treatment was omitted and the mice received either vehicle or cisplatin (*see* **Subheading 3.2.**). Treatment (systemic antisense oligonucleotide, 25 mg/kg of an equimolar mixture of antisense JNK1 and antisense JNK2 termed combined-antisense JNK1 + JNK2, or a scrambled-sequence oligonucleotide or vehicle phosphate-buffered saline [PBS] alone) was initiated on development of readily visible and palpable tumors (**Fig. 5**). Every seventh day, antisense treatment was omitted and a subgroup of 15 animals receiving the combined-antisense treatment also received cisplatin, i.e., the same dose of cisplatin and timing as for the p53 treatment regimen. As a control, a separate group of animals received cisplatin alone. To confirm that systemic antisense treatment led to inhibition of JNK activity, JNK activity of tumor extracts was determined 18 h after antisense treatment. These studies demonstrated an 89% reduction in steady-state tumor JNK activity in tumors of antisense-treated animals compared with the activity of PC-3 cells, and an 80% reduction compared with the tumor JNK activity of scrambled sequence oligonucleotide-treated animals (**Fig. 5**).

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Fig 5

We observed that treatment with antisense oligonucleotides either antisense JNK2 or combined-antisense treatment—led to marked inhibition of tumor growth, and these results were superior to those for cisplatin alone. When the growth curves in **Fig. 5** were integrated, it was found, e.g., that combined treatment led to 78% inhibition whereas cisplatin treatment led to 47% tumor growth inhibition. These results are significantly less ($p < 0.002$) than those for controls (vehicle alone, scrambled-sequence oligonucleotide) and are significantly different from each other ($p < 0.02$; analysis of variance). The effect of antisense JNK can be attributed largely to antisense JNK2 (**Fig. 5**). The domi-

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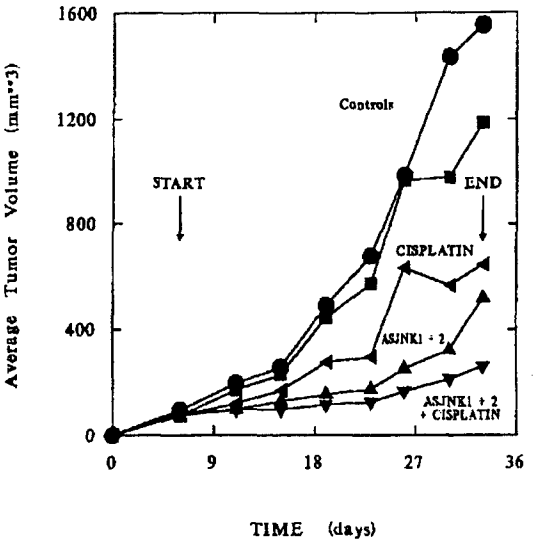

Fig. 5. Application of antisense JNK and cisplatin chemotherapy to established xenografts of PC-3 human prostate carcinoma cells. Groups of 10–11 or 15 athymic female mice that had been inoculated with PC-3 cells and allowed to develop visible tumors were started on treatment ("start" arrow) of either one of the indicated antisense oligonucleotide solutions by daily IP injection or cisplatin weekly or a control consisting of vehicle alone or a scrambled sequence oligonucleotide. The average size of the tumors for each treatment group is plotted from the time of inoculation to the end of treatment ("end" arrow). The percentage of inhibition of growth was calculated by integrating each curve and expressing the result as 100 × [1-(growth/growth of control)]. These experiments were carried out in parallel with the p53 adenovirus-treated PC-3 tumors.

nance of JNK2 has been observed in human lung carcinoma cells *(22)* and in a series of nine human prostate carcinoma cell lines *(26)*. When antisense cisplatin treatments were combined, growth inhibition was further enhanced to 89% of maximum growth inhibition (**Fig. 5**). Thus, these in vivo studies suggest that it may be possible both to inhibit growth and to sensitize solid tumors to chemotherapy by eliminating the JNK pathway.

1.5. Combined Inhibition of Jun Kinase and Restoration of p53

The approaches described here have implications for therapeutic approaches targeting the DNA damage response in tumor cells. Upregulation of the DNA repair machinery may accompany tumor progression and the development of

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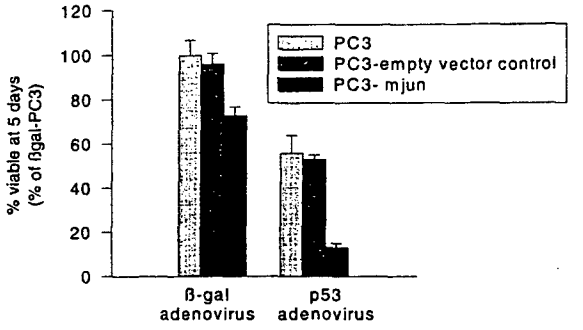

Fig. 6. Cells modified to express mutant jun are sensitized to p53. Relative 4-d growth following Ad p53 treatment or Ad–13gal treatment (control) of PC-3 prostate carcinoma cells (parental), PC-3 empty vector transduced cells, and PC-3 mutant Jun-expressing cells (PC3-mjun). Growth was assayed using the MTT assay.

drug resistance. This process may involve, in part, AP-1 and ATF2/CREB-regulated DNA synthesis and repair genes. In fact, earlier studies by Scanlon et al. *(27)* demonstrated that downregulation of AP-1 activity using a c-fos ribozyme was effective at sensitizing ovarian carcinoma cells to cisplatin, and this correlated with downregulation of DNA polymerase β, topoisomerase I, and thymidylate synthetase, all known to have AP-1 sites in their promoter regions *(27)* (**Table 2**).

Independent of JNK DNA damage response, loss of the p53 tumor suppressor contributes to resistance to DNA-damaging therapies by removing an important component of the DNA damage recognition machinery. Because p53 induces apoptosis in response to the level of DNA damage, it is likely that the success or failure of DNA repair contributes to the suppressive effects of p53. We therefore hypothesized that cells in which the JNK pathway was inhibited would show greater growth inhibition following exposure to p53 adenovirus. As shown in **Fig. 6**, this is indeed the case. In this experiment, PC-3 prostate cancer cells expressing mutant Jun were much more growth suppressed following treatment with p53 adenovirus in 7-d 96-well growth assays than were parental cells or cells modified with empty vector.

Fig 6

These results support the combined application of p53 gene therapy along with antisense therapy to inhibit the JNK pathway, or some downstream target of the pathway. When used in combination with conventional chemotherapies such as cisplatin, the combined approach, which can be foreseen today, may extend the application of conventional treatments to prostate cancer and provide a new strategy for treatment of therapy-resistance advanced disease.

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2. Materials

1. Adenoviral vectors (Deborah Wilson, Introgen Therapeutics, Houston, TX) (*see* **Note 1**).
2. Antisense phosphorothiates dexyoligonucleotides (*see* **Note 2**; *see* also **ref. *23***):
   a. JNK1: 5'-CTCTCTGTAGGCCCGCTTGG-3'.
   b. JNK2: 5'-GTCCGGGCCAGGCCAAAGTC-3'.
3. Delbecco's modified minimum medium supplemented with 10% fetal calf serum (Irvine Scientific, Irvine, CA). PC-3 human prostate carcinoma cells, as well as other cell lines used in in vitro assays (T47D breast cancer cells, DLD-1 colon cancer cells, and T98G glioblastoma cells) were grown at 37°C in an environment of 10% CO_2.
4. Animals: 5- to 6-wk athymic female postweaning Harlan Sprague-Dawley mice (Harlan, Indianapolis, IN).
5. 10X PBS: 2 g of anhydrous KH_2PO_4, 11.4 g of anhydrous Na_2HPO_4, 2 g of KCl, 80 g of NaCl. Make up the volume to 1 L with H_2O. Dilute 1:10 before use.
6. X-gal staining solution: 1 mg/mL of 5-bromo-4-chloro-3-indoyl-β-D-galactopyrano-side(X-gal), 5 m*M* potassium ferricyanate, 5 m*M* potassium ferrocyanate, 2 m*M* $MgCI_2$ in PBS.
7. *Taq* polymerase (Qiagen).
8. 250 μ*M* dNTPs (Pharmacia Biotech.).
9. PCR Reaction mix: 25-μL reactions contain 0.03–0.25 μg of DNA, 25 pmol each of forward and reverse primer, 250 μ*M* dNTPs (Pharmacia Biotech.), 1.25 U *Taq* polymerase (Qiagen), 1X buffer (Qiagen), Solution Q (Qiagen). The deoxyribonucleotide pruners are 5'-TGGGATTACACGTGTGAACCAACC-3' and 5'-GATCCACAGTCTGCCTGAGTCACT-3', respectively, with a 5' nested primer of 5'-CCTAGAAAGCACATGGAGAGCTAG-3' (*see* **Note 6**).
10. CAT assay reaction mix (per reaction): 70 μL of cell lysate, 30 μL of 5 m*M* chloramphenicol, 0.4 μL of ^{3}H-acetylCoA (200 mCi/mmol; NEN®Life Science), 0.6 μL (4.4 μg/μL) of nonradioactive acetylCoA (Pharmacia Biotech.).
11. Whole-cell extract (WCE) buffer: 20 m*M* HEPES, pH 8.0; 75 m*M* NaCl; 2.5 m*M* $MgCl_2$, 0.05% (v/v) Triton X-100, 0.5 m*M* dithiothreitol (DIT); 20 m*M* β-glycerophosphate; 0.1 m*M* Na_3VO_4; 2 μg/mL leupeptine, 100 μg/mL paramethylsulfonyl fluoride.
12. JNK assay buffer: 20 m*M* HEPES, pH 7.7; 20 m*M* $MgCl_2$, 20 m*M* β-glycerophosphate; 20 m*M* *p*-nitrophenyl phosphate; 0.1 m*M* Na_3VO_4, 2 m*M* DTT; 20 μ*M* adenosine triphosphate (ATP); 5 μCi of [λ^{32}P] ATP.
13. Oligonucleotide transfection (lipofection) solution: Mix 10 μg/mL of lipofectin (Gibco-BRL, Gaithersburg, MD) reagent in MEM (Gibco-BRL, Gaithersburg, MD) with an equal volume of oligonucleotide solution, incubating this mixture at room temperature for 15 min and diluting it with lipofectin solution to a final oligonucleotide concentration of 0.4 μ*M*.
14. X-gal staining solution: 1 mg/mL 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, 5 m*M* potassium ferricyanate, 5 m*M* potassium ferrocyanate, 2 m*M* MgCt in PBS.

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15. Lipofection solution for plasmid transfection: Prepare a 1 μg/μL stock of DOTAP lipofection reagent (Boehringer Mannheim, Indianapolis, IN). Prepare the mix (per well) of a 24-well tissue culture plate as follows:
    a. Plasmid/HEPES mix: 1.25 μL (1 μg/μL) of plasmid + 11.25 μL 5 m*M* HEPES, pH 7.8.
    b. DOTAP/HEPES mix: 7.5 μL of DOTAP (1 μg/μL) + 17.5 μL of 5 m*M* HEPES, pH 7.8.

    Mix the entire contents of (a) and (b) together, and incubate for 30 min at room temperature.
16. Cisplatin (diaminodichloro *cis*-platinum): Aqueous Platinol (Bristol Myers Squibb).

3. Methods

3.1. 96-Well Growth Assay

1. Plate cells in complete medium 1 day prior to vector treatment in 24-well tissue culture plates so that their density at the time of treatment is about 70–80% of confluence.
2. Treat the cells with Adp53 or Ad Egal for 2 to 3 h (100 PFU/cell).
3. Incubate the cells for additional 2 d in one of the Ad-βgal-treated wells.
4. Remove the medium and wash the wells two times with PBS.
5. Fix the cells by overlaying with a solution containing 3.7% parafomaldehyde (v/v) in PBS for mm.
6. Wash the wells with PBS and overlay with X-gal staining solution.
7. Incubate the cells at 37°C overnight to allow development of blue stain in β-galactosidase-expressing cells for estimating the infection efficiency.
8. Following treatment with vector, plate the cells at low density (1000 cells/well) in 96-well plates (*see* **Note 4**). Treat (in triplicate) with drug (e.g., cisplatin) or antisense (*see* **Subheading 3.6.**) for 1–4 h depending on the drug, followed by incubation for an additional 5–7 d (*see* **Note 1**).
9. Incubate the cells for an additional 6 d and score viability by adding the tetrazolium dye MTT for 1 h and determining the A260 of the formazan product as described by the manufacturer (Promega) (*see* refs. *9* and *23*).

3.2. Subcutaneous Tumor Model in Nude Mice

1. Inoculate 5×10^6 PC-3 prostate cancer cells (mutant p53) subcutaneously on the back of nude mice by injecting 100 μL of a cell suspension in PBS at 5×10^7 cells/mL.
2. Allow tumors develop 5 d to a size of 50–100 mm^3.
3. Estimate tumor volume by measuring the length and width of the tumor with a caliper and calculate using the following formula: volume = $\pi/6$ (length × $width^2$).
4. Initiate treatment (designated day 1) and monitor tumor volume every 2 to 3 d (*see* **Note 2**).
5. For oligonucleotide treatment, inject mice intraperitoneally at 25 mg/kg and based on the average weight of all mice by daily ip injection with a solution

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at a concentration yielding the total dose in 0.2 mL of PBS. Treat control animals with either vehicle alone or the scrambled-sequence control oligonucleotide.

6. For cisplatin treatment, inject mice intraperitoneally at 0.4 mg/kg once per week. For combined antisense JNK cisplatin treatment, inject antisense JNK oligodoxynucleotides for 6 d and cisplatin on the day 7.

3.3. Analyses of DNA Repair by PCR Stop Assay

1. Prepare genomic DNA from about 5×10^5 cultured cells using the QlAmp blood kit (Qiagen) essentially following the manufacturer's protocol, except lyse the cells directly on the plate in the presence of PBS, Qiagen protease, and lysis buffer supplied in the kit.
2. Following purification, adjust the DNA concentration to 0.25 mg/mL in sterile water and store at –20°C until use.
3. Carry out quantitative PCR as described *(32)* for the measurement of cisplatin adduct formation on specific regions of DNA (*see* **Note 4**). For each primer pair, verify that product formation is directly proportional to input template by performing a pilot experiment with serial twofold dilutions of template, followed by electrophoresis on a 1% agarose gel containing 0.5 µg/mL of ethidium bromide.
4. Quantify bands using a Kodak digital camera and analysis software or an equivalent apparatus for the integration of band intensity from photographic film and determine adduct numbers using $P = e^{-n}$ (*see* **Note 4**).
5. For the PCR reaction, use primers at a concentration of 0.03–0.25 µg per 25-µL reaction, depending on the amount of template used in the PCR reaction.
6. Carry out the PCR reaction using the following amplification program: 1 cycle of 94°C for 1 min 30 s; 25 cycles of 94°C for 1 min, 57°C, for 1 min, and 70°C for 2 min 30 s; 1 cycle of 94°C for 1 min; 57°C for 1 min, and 70°C for 7 min.
7. Use two independent templates for each treatment condition and set up each for analysis in triplicate with one template as an internal PCR control in order to generate a 270-bp fragment of the dihydrofolate reductase gene (*see* **Notes 3** and **6**).

Au: OK to add step 7?

3.4. Analysis of DNA Repair Using CAT Reporter Assay

1. Treat 100 µg of CAT reporter plasmid (in which the CAT gene is expressed from the RSV promoter), with 25 µ*M* cisplatin in 300 µL total of PBS on ice for 3 h to create cisplatin-damage reporter DNA.
2. Precipitate the DNA by adding 150 µL of 7.5 *M* NH_4Ac and 450 µL of isopropanol.
3. Leave the mixture at –20°C for 1 h.
4. Centrifuge the DNA at 14,000*g* for 30 min.
5. Perform two washes with 70% ethanol, followed by a final wash in absolute ethanol.
6. Resuspend the DNA in sterile H_2O and use for transfections.
7. Process untreated DNA the same way but without the addition of cisplatin, and use this plasmid preparation as a control.

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8. Plate the cells in 24-well plates the day prior to transfection so that their density at the time of transfection is 50% (about 10^5 cells/well).
9. Set up transfections in triplicate wells with either 1–1.25 µg of undamaged plasmid or damaged plasmid for the 24-h time point together with a second set of triplicate wells transfected for the 48-h time point, using the DOTAP liposomal transfection reagent (*see* **Subheading 2.5.9.**).
10. Add 500 µL of medium to the entire contents of **step 7** and mix well.
11. Wash the cells in the well of a 24-well plate twice with 1X PBS.
12. Add 537.5 µL of transfection (**step 8**) to the well.
13. Incubate the reaction overnight in the 37°C, 10% CO_2 incubator.
14. Aspirate off the transfection mix and wash the cells twice with 1X PBS.
15. Aspirate off the PBS and replace with 1 mL of fresh complete culture medium.
16. Incubate for 24–48 h at 37°C, 10% CO_2.
17. Remove the medium, wash the cells twice with PBS, and store the plate dry at –70°C until required.

Au: There is no 2.5.9. Please clarify.

3.5. CAT Assay

1. Thaw the plates from **step 16** in **Subheading 3.4.** for 5 min at 37°C.
2. Add 100 µL of 1X Reporter Lysis Buffer (Promega) to each well of a 24-well plate.
3. Shake the plate vigorously for 15 min.
4. Transfer the entire 100 µL of lysate to microcentrifuge tubes.
5. Spin the lysates for 2 min at 14,000*g* (4°C).
6. Transfer the supernatants to fresh tubes.
7. Heat the supernatants for 10–15 min at 65°C to destroy deacetylase activity. If particulate material is present after this heating step, centrifuge again and collect the supernatants.
8. Measure the protein concentration of each sample using the Bio-Rad Protein Assay (Bio-Rad), following the manufacturer's instructions.
9. Equalize the protein concentration of the samples using 1X Reporter Lysis Buffer (Promega). Add 31 µL of CAT reaction mix to 70 µL of the samples.
10. Incubate the mix at 37°C for 2 h.
11. Add the entire sample to 1 mL of 7 *M* urea.
12. Add 1 mL of toluene:PPO (8 g of PPO [2.5-diphenyloxazole]/mL toluene). Shake well and count using a scintillation counter. The resulting counts are proportional to accumulated CAT expression and, therefore, total CAT activity.

Au: OK? PPO?

3.6. Antisense Oligonucleotide Transfection

1. Wash 70% confluent cultures (24 h after plating) in tissue culture plates twice with PBS.
2. Incubate the cells with lipofectin-0.4 µM oligonucleotide solution (*see* **Subheading 2**) at 37°C in 10% CO_2 from 4 h to overnight (*see* **Note 7**).
3. Following lipofection, wash the cells once with serum-free MEM and continue the culture in complete medium for 24 h prior to JNK assay (*see* **Subheading** 3.7) or 4 d prior to growth assay *(9)* (*see* **Note 7**).

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3.7. JNK Assay

1. Transfect the cells with oligonucleotides as described in **Subheading 3.5.**
2. Wash the cells with ice-cold PBS and suspend in WCE buffer.
3. Determine the protein concentration of the cell extracts by the Bradford dye method (Bio-Rad) (*see* **Note 8**).
4. Carry out the kinase assay as follows ***(28,29)***:
   a. Mix 50 µg of WCE with 10 µg of GST-c-Jun (1-223) for 3 h at 4°C (*see* **Note 9**).
   b. Wash four times and incubate the beads with 30 µL of kinase reaction buffer for 20 min at 30°C.
5. Stop the reaction by adding of 20 µL of Laemmli sample buffer.
6. Elute the phosphorylated GST-c-Jun protein by boiling the sample for 5 min.
7. Resolve the components by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (*see* **Note 8**).
8. Quantify the ^{32}P-phosphorylated GST-c-Jun by digitization and integration of the respective "band" values of the autoradiograph of the dried gel (*see* **Notes 3** and **10**).

4. Notes

1. These vectors are replication-defective adenoviral recombinants in which the early region genes E1A and E1B required for viral replication have been deleted and replaced with an expression cassette containing the transgene of interest. In the case of the p53 adenovirus (Adp53), the expression cassette consisted of the human wild-type p53 coding sequence, flanked by the cytomegalovirus (CMV) promoter, and the simian virus 40 (SV40) polyadenylation signal. In the case of the control adenovirus (Adβgal), the expression cassette consisted of the bacterial β-galactosidase coding sequence flanked by the CMV promoter and SV40 polyadenylation signals. In the case of the control adenovirus (AdLuc), the expression cassette consisted of the firefly luciferase coding sequence flanked by the Rous sarcoma virus promoter and SV40 polyadenylation sequences. Viral stocks were stored at –70° C and repeated freezing and thawing were avoided. The concentrations of the stocks, expressed in plaque-forming units per milliliter were 1 to 2×10^{11}.
2. The sequences were determined in preliminary studies utilizing a messenger walk procedure as described (*see* **refs. *28*** and ***29***). Since in vivo studies require large amounts of oligonucleotide, a single "scrambled sequence" control sequence was chosen consisting of a scrambled 20-nt sequence previously used as a control in the analysis of antisense protein kinase Ca: 5' TCGCATCGACCCGCCCACTA-3'. Both ASJNK sequences contain a single CpG sequence thought to have potential immunostimulatory properties that, therefore, may influence xenograft growth by immunologic mechanisms. The control oligonucleotide used here has a nucleotide composition closely approximating the average of antisense JNK1 and JNK2 but contains three CpG dinucleotide sequences thereby providing a control for the potential influence of CpG sequences. All three oligonucleotides were prepared and purified as previously described ***(34,35)***.

3. The 0.15-kb segment of genomic DNA amplified by use of the nested primer sustains undetectable levels of DNA damage under our conditions and serves as an internal PCR control and the basis for normalization of the amount of amplification of the 2.7-kb fragment. The nested amplification product varies by <5–10% among the various templates that we have used.
4. Wells that do not receive drug serve as a control to which drug-treated wells are compared. Example drug treatments are as follows: 5-fluorouracil (Adrucil™, Pharmacia Biotech.), 10 m*M* for 1 h; doxorubicin (doxorubicin hydrochloride, Aldrich), 3.7 μ*M* for 1 h, cisplatin (Platinol™, Bristol-Myers Squibb), 30 μ*M* for 1 h for PC-3 and 20 μ*M* for 1 h for T98G.
5. Because *Taq* polymerase is blocked at cisplatin adducts, the relative efficiency of PCR amplification of genomic DNA from cisplatin-treated vs control cells drops in proportion to platination levels. The relative PCR efficiency is equal to the frequency of undamaged strands, *P*, within a population. *P* is related to the average number of cisplatin adducts, *n*, per fragment, by the Poisson formula $P = e^{-n}$, or $-(\ln P) = n$. A drop in the PCR signal to 0.6 of control would therefore reflect an average cisplatin adduct density of –(in 0.6) = 0.51 adducts per fragment. PCR signals ranging from 0.9 to 1.0 of control are generally indistinguishable from the control, owing to standard deviations in the range of ±0.1. Since a PCR signal equal to 0.9 of control reflects an adduct density of 0.1 adduct per fragment, we consider that the assay is not sensitive to adduct densities of <0.1 adduct per fragment. In most cases with genomic DNA from cisplatin-treated cells, the assay requires PCR amplifying a fragment of about 2 to 3 kb long.
6. An example treatment consisted of ip administration if cisplatin (Platinol) on d 1 and 8 at a dose of 4 mg/kg (LD10), i.e., 88 μL/22 g mouse. The dose corresponds to approx 20% of the IC50 of cisplatin for mice and is the maximum tolerable dose for a repeated weekly treatment. Vector (Adp53 or AdLuc) was administered intratumorally at a dose of 10^8 PFU/injection on d 3, 5, and 7 and again on d 10, 12, and 14. Vector was diluted into sterile cold PBS prior to injection so that injection volumes were 100 μL.
7. Generally a 4-h exposure is required for the reduction in target JNK rnRNA by >80%. This is observed after 24 h of lipofection as judged by western analysis. Resistant cell types are treated for longer periods, and this approach *is* favored over increasing the oligonucleotide concentration or altering the oligonucleotide:lipofectamine ratio.
8. Typically all preparations yield quite similar protein concentrations, but volumes should be used so as to provide equal amounts of total cellular protein in all samples prior to subsequent analysis.
9. Prepare in advance GST-c-Jun fusion proteins by expression and purification (Qiagen plasmid DNA purification system kit) from *Escherichia coli* and by the addition of Glutathione Sepharose® 4B beads (Pharmacia Biotech).
10. For digitization software that provides interactive designation of the bands by, e.g., drawing boxes around the region to be digitized. If not automated, subtract background by using half-sized boxes placed exactly above and below

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the band in question or by subtracting one half of the sum of same-sized boxes placed above and below the band in question. Subtract background values in all cases, thereby yielding "net" band values. Determine the relative JNK activity by normalizing the resulting net band values by division by similar results for control cases such as the scrambled-sequence or mock transfected cell case.

Acknowledgments

This work was supported in part by grants NCI CA69546 (R.A.G.), NCI CA63783 (D.A.M.), and NCI CA76173 (D.A.M.) from the U.S. Public Health Service; by the U.S. Army Breast Cancer Research Program DAMD17-96-1-6038 (R.A.G.); Introgen Therapeutics, Inc. (R.A.G.); and the Fellowship Program of the Sidney Kimmel Cancer Center. We thank Kluwer Academic/Plenum Publishers, Inc. (New York, Boston, Dordrecht, London, Moscow) for permission to reprint Figs. 1 and 2 and Tables 1 and 2 from "Cancer Gene Therapy: Past Achievements and Future Challenges" (Advances in Experimental Medicine and Biology, Vol. 465), N. Habib, ed. (2000).

We thank Angela Narehood for excellent editorial assistance.

References

1. Fischer, D. S., Knobf, M. T., and Durivage, H. J. (1993) *The Cancer Chemotherapy Handbook,* 4th ed, Mosby, St. Louis.
2. Cantrell, J. E., Hart, R. D., Taylor, R. F., and Harvey, J. H. Jr. (1987) Pilot trial of prolonged continuous-infusion 5-fluorouracil and weekly cisplatin in advanced colorectal cancer. *Cancer Treat. Rep.* **71,** 615–618.
3. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird C. C., Hooper M. L., and Wyllie, A. H. (1993) Thymocyte apoptosis induced by pS3-dependent and independent pathways. *Nature* **362,** 849–852.
4. Gjerset, R. A., Turla, S. T., Sobol, R. E., Scalise, J. J., Mercola, D. M., Collins, H., and Hopkins, P. J. (1995) Use of wild-type pS3 to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p35. *Mol. Carcinog.* **14,** 275–285.
5. Dorigo, O., Turla, S. T., Lebedeva, S., and Gjerset, R. A. (1998) Sensitization of rat glioblastoma multiforme to cisplatin *in vivo* following restoration of wild-type pS3 function. *J. Neurosurg.* **88,** 535–540.
6. Lotem, J. and Sachs, L. (1993) Hematopoietic cells from mice deficient in wild-type pS3 are more resistant to induction of apoptosis by some agents. *Blood* **82,** 1092–1096.
7. Lowe, S. W., Bodis, S., McClatchy, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. (1994) p53 status and the efficacy of cancer therapy *in vivo. Science* **266,** 807–810.
8. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. (1993) p53-mediated apoptosis modulates the cytotoxicity of anti-cancer agents. *Cell* **74,** 957–967.

Job: STARKEY 774-6 (MiMB 97-6.0)
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Pub Date: 2000

Operator: RH
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Revision: Straight to pgs 495–520

9. Potapova, O., Haghighi, A., Bost, F., Liu, C., Birrer, M. J., Gjerset, R., and Mercola, D. (1997) The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. *J. Biol. Chem.* **30,** 14,041–14,044.
10. Asgari, K., Sesterhenn, I. A., McLeod, D. G., Cowan, K., Moul, J. W., Seth, P., and Srivastava, S. (1997) Inhibition of the growth of pre-established subcutaneous tumor nodules of human prostate cancer cells by single injection of the recombinant adenovirus p53 expression vector. *J. Cancer* **71,** 377–382.
11. Ko, S. C., Gotoh, A., Thalmann, G. N., Zhau, H. E., Johnston, D. A., Zhang, W. W., Kao, C., and Chung, L. W. (1996) Molecular therapy with recombinant p53 adenovirus in an androgen-independent, metastatic human prostate cancer model. *Hum. Gene Ther.* **7,** 1683–1691.
12. Swisher, S. G., Roth, J. A., Nemunaitis, et al. (1999) Adenovirus-mediated pS3 gene transfer in advanced non-small cell lung cancer. *J. Natl. Cancer Inst.* **91,** 763–771.
13. Kauczor, H. U., Schuler, M., Heussel, C. P., von Weymarn, A., Bongartz, G., Rochlitz, C., Huber, C., and Thelen, M. (1999) CT-guided intratumoral gene therapy in non-smallcell lung cancer. *Eur. Radiol.* **9,** 292–296.
14. Roth, J. A. (1998) Restoration of tumor suppressor gene expression for cancer. *Forum* **8,** 368–376.
15. Schuler, M., Rochlitz, C., Horowitz, J. A., Schelgel, J., Perruchoud, A. P., Kommoss, F., Bolliger, C. T., Kauzor, H. U., Dalquen, P., Fritz, M. A., Swanson, S., Herrmann, R., and Huber, C. (1998) A phase I study of adenovirus-mediated wild-type pS3 gene transfer in patients with advanced non-small cell lung cancer. *Hum. Gene Ther.* **9,** 2075–2082.
16. Roth, J. A., Swisher, S. G., Merritt, J. A., et al. (1998) Gene therapy for non-small cell lung cancer: a preliminary report of a phase I trial of adenoviral p53 gene replacement. *Oncology* **25,** 33–37.
17. Zwaka, R. M. and Dunlop, M. G. (1998) Gene therapy for colon cancer. *Hematol. Oncol. Clin. North Am.* **12,** 595–615.
18. Clayman, G. L., el-Naggar, A. K., Lippmann, S. M., et al. (1998) Adenovirus-mediated p53 gene transfer in patients with advanced recurrent head and neck squamous cell carcinoma. *J. Clin. Oncol.* **16,** 2221–2232.
19. Bost, F., Potapova, O., Liu, C., Zhang, Y.-M., Charbono, W., Dean, N., McKay, R., and Mercola, D. (1999) High frequency regression of established human prostate carcinoma PC-3 xenografts by systemic treatment with antisense Jun kinase. *The Prostate* **38,** 320, 321.
20. Loda Magi-Galluzzi, C., Mishra, R., Fiorentino, M., Montironi, R., Yao, H., Capodieci, P., Wishnow, K., Kaplan, I., Stork, P. J., and Loda, M. (1997) Mitogen-activated protein kinase phosphatase 1 is overexpressed in prostate cancers and is inversely related to apoptosis. *Lab. Invest.* **76,** 61–70.
21. Jennerwein, M. M. and Eastrman, A. (1991) A polymerase chain reaction-based method to detect cisplatin adducts in specific genes. *Nucleic Acids Res.* **19,** 6209–6214.

Job: STARKEY 774-6 (MiMB 97-6.0)
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Pub Date: 2000

Operator: RH
Date: 1/3/01
Revision: Straight to pgs 495-520

51. Gately, D. P., Jones, J. A., Christen, R., Barton, R. M., Los, G., and Howell, S. B. (1994) Induction of the growth arrest and DNA damage-inducible gene GADD153 by cisplatin in vitro and in vivo. *Br. J. Cancer* **70,** 1102–1106.
52. Delmastro, D. A., Li, J., Vaisman, A., Solle, M., and Chaney, S. G. (1997) DNA damage inducible-gene expression following platinum treatment in human ovarian carcinoma cell lines. *Cancer Chemother. Pharmacol.* **39,** 245–253.
53. Kolodner, R. D., Hall, N. R., Liptord, J., Kane, M. F., Rao, M. R., Morrison, P., Wirth, L., Finan, P. J., Burn, J., and Chapman, P. (1994) Structure of the human MSH2 locus and analysis of two Muir-Torre kindreds for msh2 mutations. *Genomics* **3,** 516–526.
54. Lamerdin, J. E., Montgomery, M. A., Stilwagen, S. A., Scheidecker, L. K., Tebbs, R. S., Brookman, K. W., Thompson, L. H., and Carrano, A. V. (1995) Genomic sequence comparison of the human and mouse XRCC1 DNA repair gene regions. *Genomics* **25,** 547–554.
55. Leach, F. S., Nicolaides, N. C., Papadopoulos, N., et al. (1993) Mutations of a mutS homologin hereditary nonpolyposis colorectal cancer. *Cell* **75,** 1215–1225.
56. Iwakuma, T., Shiraishi, A., Fukuhara, M., Kawate, H., and Sekiguchi, M. (1996) Organization and expression of the mouse gene for DNA repair methyltransferase. *DNA Cell Biol.* **15,** 863–872.
57. Lefebvre, P., Zak, P., and Laval, F. (1993) Induction of 06-methylguanine-DNA methyltransferase and N3-methyladenine-DNA-glycosylase in human cells exposed to DNA-damaging agents. *Cell Biol.* **12,** 233–241.
58. Scherer, S. J., Seib, T., Seitz, G., Dooley, S., and Welter, C. (1996) Isolation and characterization of the human mismatch repair gene hMSH2 promoter region. *Hum. Genet.* **97,** 114–116.
59. Mello, J. A., Acharya, S., Fishel, R., and Essigmann, J. M. (1996) The mismatch-repair protein hMSH2 binds selectively to DNA adducts of the anticancer drug cisplatin. *Chem. Biol.* **3,** 579–589.
60. Dalton, T. P., Li, Q., Bittel, D., Liang, L., and Andrews, G. K. (1996) Oxidative stress activates metal-responsive transcription factor-1 binding activity: occupancy *in vivo* of metal response elements in the metallothionein-I gene promoter. *J. Biol. Chem.* **271,** 26,233–26,241.
61. Gjerset, R. and Mercola, D. (2000) Sensitization of tumors to chemotherapy through gene therapy, in *Advances in Experimental Medicine and Biology* (Habib, N., ed.), Plenum, New York, pp. 273–292.

In press: Encyclopedia of Cancer, 2nd edition, J. Bertino, ed.

THERAPEUTIC APPLICATIONS OF A P53 GENE THERAPY VECTOR, Ad5CMV-*p53*

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OUTLINE

I. DEFINITION OF SUBJECT.

The p53 tumor suppressor protein is a 53 K dalton transcription factor that plays a key role in inducing cell cycle arrest and DNA damage-induced programmed cell death (apoptosis) in tumor cells. The highly conserved sequence of p53, and the finding that it is frequently lost or mutated in most types of cancer, suggests a central role of p53 in the process controlling tumorigensis. Over the past 10 years, p53 has been the focus of a large body of research, which has led to an expanded and refined understanding of cancer on the one hand, and provided us, on the other hand, with new therapeutic strategies to cancer treatment. P53 gene therapy is presently being tested for therapeutic efficacy in clinical trials of several cancers, including those of the head and neck, prostate, breast, and lung (see Levine, 1993 for review).

II. LOSS OF P53 AND THE PATHOGENESIS OF CANCER

Initially p53 was identified as an SV40 T antigen-binding protein in cells transformed with the Simian Virus 40 (SV40) tumor virus. This observation, together with the frequent over-expression of p53 in murine and human tumor cells, led initially to the erroneous classification of p53 as an oncogene. Some early cellular transformation experiments corroborated this notion by showing that p53 gene transfer could promote transformation and immortalization of murine fibroblasts in cell culture. However, it was later discovered that it was the mutated form of p53 that had been used in these cellular transformation assays. Wild-type p53 had no ongogenic activity and was instead a potent tumor suppressor that was profoundly growth suppressive in tumor cells and inhibitory to cellular transformation. The accumulation of p53 protein that was often observed in

tumor cells resulted from mutations that conferred enhanced stability to the protein and increased its half-life from minutes for the wild-type protein to several hours for the mutant protein. But neither the stabilized form of the mutant protein nor T-antigen-bound wild-type p53 could carry out wild-type p53 functions.

Various lines of evidence suggest that an important component of wild-type p53's tumor suppressor function involves DNA damage recognition and induction of apoptosis. Following DNA damage recognition, p53 is stabilized and activated as a transcription factor, which in turn leads to the expression of p53 target genes involved in apoptosis. Endogenous DNA damage may be a fundamental feature of the cancer cell, arising as a consequence of genome instability, a hallmark of cancer. Because this endogenous damage could serve as a trigger for p53-mediated apoptosis, the loss of p53, through gene deletion or mutation, would provide a survival advantage for the cancer cell by enabling escape from apoptosis. Such a model is consistent with studies in transgenic mice predisposed to mammary tumors, where loss of p53 was correlated with increased genome instability and aneuploidy (Donehower, 1995).

III. LOSS OF P53 AND THERAPY RESISTANCE

While loss of p53-mediated DNA damage-induced apoptosis would favor the outgrowth of genomically unstable cancer cells, it also promotes resistance to a variety of anticancer agents that act, at least in part, through the induction of DNA damage. For example, hematopoietic cells from p53-null transgenic mice (Lotem, 1993), as well as E1A-transformed fibroblasts from p53-null transgenic mice (Lowe, 1993), were found to be much more resistant to DNA damaging treatments than were the corresponding cells from

normal mice. Furthermore, the growth inhibitory properties of a large panel of anticancer agents were seen to correlate with the p53 status of the tumor cells. In general, tumor cells that expressed wild-type p53 were more responsive to most of these agents than were tumor cells with mutated or deleted p53. An exception to this general observation was found with the class of agents known as anti-mitotics, which target the mitotic apparatus rather than DNA (O'Connor, 1997).

These observations are particularly important in light of the fact that resistance to conventional chemotherapeutic drugs is still a major obstacle to successful treatment of cancer, and is likely to account for treatment failure in about half of all cancers. To the extent that loss of wild-type p53 function contributes to drug resistance, it may be possible, using p53 gene replacement strategies as described below, to reverse or alleviate drug resistance, enabling us to fully exploit the potential of standard chemotherapies. A variety of gene transfer approaches are being developed for clinical application of gene therapy, including viral and non-viral approaches. Here we focus on approaches that use adenovirus, chosen for its unparalleled gene transfer efficiency to a wide range of tissue types, and for its relative safety and minimal toxicity.

IV. GENE REPLACEMENT STRATEGIES INVOLVING ADENOVIRUS.

The first representative of the adenovirus family was isolated in the early 1950s from the adenoids of individuals suffering from upper respiratory tract infections. More than 100 different species of adenovirus have now been identified, and they are associated with a range of illnesses of varying severity depending on type. However, one of the most studied of these virus species, human adenovirus type 5, primarily causes mild cold-like

symptoms and is not associated with serious pathogenicity. For this reason, adenovirus type 5 has attracted interest as a vector for cancer gene therapy.

Adenoviruses are characterized by a capsid consisting of several well defined structural proteins, including the main structural proteins termed hexon (surrounded by 6 other capsid proteins), penton (surrounded by 5 other capsid proteins, and fiber (which mediates attachment to the target cell). The assembled capsid is icosahedral in shape (20 faces) and measures about 900 angstroms in diameter. Adenoviral genomes are linear, double-stranded DNA molecules. A great deal has been learned about their genome structure and the functions of the various viral gene products, and genetically engineered versions of adenovirus suitable for gene transfer applications have been generated. The vectors based on adenovirus type 5 presently in use in both preclinical and clinical studies lack specific genes required for virus replication. Although these viruses are still able to enter their target cell, delivering the human gene of interest, they are unable to replicate their DNA and produce progeny, and therefore are disabled with regard to pathogenicity.

In addition to their lack of serious pathogenicity and their high efficiency of gene transfer, adenoviruses are attractive for therapeutic applications because of their broad host cell specificity. Furthermore, adenoviruses are relatively easy to prepare in high titer on an industrial scale. Long-term transgene expression is not anticipated with adenovirus, as the vectors are impaired for replication in the target cell, and the vector DNA does not integrate into the host cell genome. While transient expression could constitute a disadvantage for certain gene therapy applications, it is not a limitation for cancer gene therapy, where the endpoint of therapy is cell death. Another potential limitation of

adenovirus, namely its immunogenicity, has not been a limitation for application in clinical trials of cancer patients to date (see below).

V. PRECLINICAL APPLICATIONS OF P53-ADENOVIRUS.

Numerous in vitro studies carried out on tumor cells in culture have demonstrated that restoration of p53 activity in tumor cells is growth suppressive in itself, and this suppression is enhanced in the presence of any of a wide variety of chemotherapeutic drugs, including cisplatin, doxorubicin (Adriamycin®), and 5-fluorouracil. In some cases, the suppressive effect of the combination of p53 gene replacement and chemotherapeutic drug treatment is greater than the sum of the suppressive effects of each agent used singly, suggestive of mechanistic synergy, and is consistent with the DNA damage-dependent mechanism by which p53 is believed to act (Gjerset, 1995). One example of this is shown in Figure 1, where the T47D breast cancer cell line was treated with p53 adenovirus under conditions where approximately 70% of the culture expressed wild-type p53, with the DNA damaging chemotherapeutic drug, cisplatin, alone, or with a combination of the two. Six days post treatment the viability of cells treated with the individual agents had dropped to about 70% of that of the control cells. However, when cells were treated with a combination of p53 adenovirus and cisplatin, the viability of the cells dropped to 10% of that of the control cells, a value less than would be expected based on merely additive effects. These effects are not seen in normal cells, possibly because wild-type p53 has a very short half-life in normal cells. Taken together, the in vitro results support the clinical application of p53 gene therapy to suppress tumor growth and enhance tumor responsiveness to chemotherapy.

Subcutaneous tumor models in nude mice have been used to demonstrate the in vivo efficacy of p53 adenovirus for the treatment of a variety of human tumors, including breast cancer, lung cancer, colon cancer, and head and neck cancer. In these studies, p53 adenovirus administered intratumorally or regionally to pre-established tumors resulted in a marked reduction in tumor growth, which was further reduced when treatment was combined with chemotherapy. Figure 2 shows the results of a study carried out to test the efficacy of a combination of p53 adenovirus plus cisplatin for the treatment of head and neck cancer. Cisplatin is an effective and commonly used chemotherapeutic for head and neck cancer, but therapy often fails due to acquired resistance. Nude mice were implanted with Fadu head and neck tumor cells and tumors were allowed to develop to a size of about 40 mm^3. Animals were then randomized into treatment groups of 10 animals each and treatment was administered (first day of treatment was designated day 1). All groups received adenovirus (p53 adenovirus or luciferase adenovirus (control)) on days 3, 6, and 8 and again on days 17, 20, and 22. Two groups received cisplatin on days 1 and 15 by intraperitoneal injection. Tumor size was monitored at 2-3 day intervals. As shown in Figure 2, both p53 adenovirus and cisplatin had a suppressive effect when used as single agent treatments. However, the combination of p53 adenovirus and cisplatin resulted in a highly significant suppression of tumor growth relative to that seen in each of the other three treatment groups ($p<0.01$ by a non-parametric (Omnibus) analysis). Treatments were well tolerated by the animals, as judged by weight measurements and histological analyses. These results suggest that the clinical application of p53 adenovirus to the treatment of head and neck cancer may be very effective in suppressing tumor growth and enhancing responsiveness to cisplatin. Similar results have been

observed using subcutaneous models for a variety of other human cancers, indicating that p53 gene therapy has broad clinical application as a tumor suppressor and therapy sensitizer.

VI. CLINICAL APPLICATIONS OF THE P53-ADENOVIRUS, Ad5CMV-*p53* (RPR/INGN 201)

a. Clinical Targets for Local/Regional Vector Administration

Many of the preclinical studies that have been done with the p53 adenoviral vector have involved direct intratumoral administration into a subcutaneous tumor in a nude mouse model. The reason for this is to maximize the exposure of the target tumor cells to the vector. The current generation adenoviral vector used for delivery of the p53 gene relies on normal adenoviral tropism in order to infect cells. Since many cell types in addition to the target tumor cells can be infected by the vector, injection into the circulation would serve to reduce the amount of vector that actually reaches these tumor cells due to the effects of dilution and uptake by non-target cells. Thus, in most of the clinical studies currently ongoing with the p53 adenovirus, Ad5CMV-*p53*, delivery of the vector to the target tumor cells is accomplished by a direct administration into a patient's tumor or into the region where a tumor is localized.

The selection of clinical targets for this type of administration has therefore focused on those cancers that would benefit from an improvement in local or regional control of tumor growth. Head and neck cancer is an excellent example of such a disease. The vast majority of patients who die with head and neck cancer die from disease in a very

localized region. Intratumoral administration in this disease is also straightforward as most of the lesions can be readily accessed for vector injection. Other tumor types that are amenable to this type of approach include brain cancer, locally advanced prostate cancer, ovarian cancer, bladder cancer, and non-metastatic stages of non-small cell lung cancer. Metastatic lesions that cause significant patient morbidity and that can be needle-accessed can also be treated in order to potentially provide patients with symptomatic improvement and possibly increased survival.

Initial Phase I studies with the p53 adenovirus, Ad5CMV-*p53,* have been conducted in two tumor types, non-small cell lung cancer (NSCLC) (Swisher, 1999; Numentaitis, 2000) and head and neck cancer (SCCHN) (Clayman, 1998). In each study the vector was administered by direct intratumoral injection into patients with advanced disease. These Phase I studies were primarily designed to determine the safety of the vector and to verify that it was able to infect and be expressed in the target tumor tissue. Each study included a dose escalation between patient cohorts; once the vector was demonstrated to be safe in patients at one dose level, the next cohort of patients was treated at a higher dose. Dose levels ranged from 10^6 to 10^{11} plaque forming units (pfu)/injection. Patients were treated by different schedules of administration in the two studies. In the non-small cell lung cancer study (Swisher, 1999), patients received a single injection of the vector once a month; this administration could then be repeated monthly as long as there was no progression of the patient's cancer. In the head and neck cancer study (Clayman, 1998), patients received six injections over a two-week period. The six-injection regimen could also be repeated monthly as long as the disease did not progress. The two treatment

regimens were each well tolerated by the patients; the major side-effects resulting from administration of Ad5CMV-*p53* were transient fever and chills and injection site pain, and in the head and neck cancer study, injection site bleeding. There was no dose-limiting toxicity found in either study. Some patients received injections for up to 6-7 months, the cut-offs for treatment that had been predefined for each study. These results demonstrated that Ad5CMV-*p53* administered by intratumoral injection had an excellent safety profile in patients with advanced cancers.

Each of these Phase I studies demonstrated that Ad5CMV-*p53* successfully transduced target tumor cells and resulted in the expression of the p53 transgene. Although patients on the trials generally had an increase in anti-adenoviral antibody titer following treatment, this did not appear to abrogate transduction as investigators demonstrated both transduction and transgene expression in biopsy material recovered from patients with elevated antibody titers. The results of these studies indicate that multiple cycles of intratumoral injection are reasonable, from both safety and transduction standpoints, to provide for longer-term patient treatment.

Additional Phase I trials have been completed for locally advanced prostate cancer (Logothetis, 1999; Sweeney, 2000) and are ongoing in additional cancer indications that are amenable to local/regional treatment approaches including bladder (Pagliaro, 2000), brain (glioblastoma multiforme) (Lang, 2000), breast, ovarian (Wolf, 2000), and bronchoalveolar carcinoma (Kubba, 2000), a type of non-small cell lung cancer. These studies are utilizing different routes of vector administration that include transrectal ultrasound-guided (TRUS), transperineal injection into the prostate (Logothetis, 1999;

Sweeney, 2000), intravesical (bladder) instillation (Pagliaro, 2000), direct injection into chest wall lesions, intraperitoneal administration (Wolf, 2000), and bronchial lavage (Kubba, 2000). As with the initial Phase I studies, endpoints for these studies are primarily safety and demonstration of transduction/expression by the various routes of administration.

b. Demonstration of clinical activity of Ad5CMV-*p53* in Phase I and Phase II clinical trials

Although the primary goal of the Phase I studies in NSCLC and SCCHN was to evaluate the safety of Ad5CMV-*p53*, measurements of the treated lesions were made before treatment and at various times following treatment in order to determine whether there was objective evidence of clinical activity. Several of the tumors injected in the Phase I studies underwent shrinkage or elimination in response to administration of Ad5CMV-*p53*. On the head and neck cancer study (Clayman, 1998), two of 33 patients had a partial response, i.e., a greater than 50% reduction in tumor size, in their treated tumors, and one patient had a complete response, or elimination, of the treated lesion. These responses occurred in both $p53^{wild\text{-}type}$ and $p53^{mutant}$ SCCHN tumors, indicating that the vector can have activity regardless of endogenous p53 status. Of the 52 patients treated on the non-small cell lung cancer study (Swisher, 1999; Nemunaitis, 2000), four had a greater than 50% decrease in the treated lesion in response to vector injection. Many other patients on the two trials had disease stabilization that lasted for 2-14 months.

Based on the promising results of the Phase I study, Phase II studies in patients with recurrent or refractory SCCHN were initiated. The largest of the Phase II studies

examined the effect of schedule on clinical activity and safety. Two schedules of administration were tested, a daily x 3 injection and the six injections over two weeks that had been used in the initial Phase I trial. Each regimen was repeated monthly until disease progressed or patients became otherwise ineligible. Preliminary results have been reported for 106 of the patients treated on the trial (Goodwin, 1999). The vector was well tolerated with side effects similar to those seen in the Phase I trial. Of the 167 lesions that were treated and evaluable for response, there were 6 and 11 that underwent complete and partial responses, respectively. As had been true in the Phase I study, responses were found in both $p53^{wild\text{-}type}$ and $p53^{mutant}$ tumors. An additional 82 lesions displayed a minor response or had disease stabilization for 3-7+ months. There was a trend towards an improved survival in patients receiving the more intensive 6-dose regimen. Phase III studies have now been initiated to determine whether administration of the vector leads to clinical benefit in patients with refractory SCCHN.

c. Combination of p53 gene therapy with conventional cancer treatment

Each of the initial Phase I studies in SCCHN and NSCLC included a second treatment arm in which a conventional cancer treatment was tested in combination with p53 gene therapy. Combination approaches are generally used for cancer treatment in order to improve the therapeutic benefit to the patient. In the non-small cell lung cancer study, patients on the second treatment arm received Ad5CMV-*p53* following a pretreatment with cisplatin. This combination had been found in preclinical studies with models of non-small cell lung cancer to lead to increased anti-tumor effects over either agent alone (Nguyen, 1996). As in the arm where patients received only Ad5CMV-*p53*, there was a

dose escalation of the vector between cohorts of patients in order to determine the safety of the combination approach. The results showed that there was no increase in toxicity due to combination treatment (Nemunaitis, 2000). This study has paved the way for further clinical studies in which the p53 adenovirus will be combined with commonly used chemotherapeutic agents to test its ability to improve therapeutic benefit.

The second arm of the Phase I head and neck cancer study involved patients whose tumors could be resected, although not as a curative procedure. Surgery, particularly in combination with radiotherapy, is a standard treatment for newly diagnosed head and neck cancer. As in the arm where patients received only Ad5CMV-*p53*, there was a dose escalation of the vector between cohorts of patients in order to determine whether vector administration could be safely combined with surgery. Results of the study showed that vector administration had no impact on wound healing and suggested that additional trials should be conducted to further explore the benefits of this approach (Clayman, 1998).

A Phase II program is ongoing in patients with NSCLC in which Ad5CMV-*p53* is being administered in combination with external beam radiation for patients with localized, inoperable NSCLC who are not eligible for chemotherapy. Patients receive three doses of Ad5CMV-*p53* during a 6-week course of radiotherapy. The study is examining the safety of the combination treatment and uses radiographic imaging and biopsy analysis to assess clinical activity. Preliminary results for the first 17 patients (Swisher, 2000) established an acceptable safety profile for the combination approach. Eleven of the 17 patients had complete or partial responses of the injected tumors. Of these, 9 also had no evidence of viable tumor cells in the biopsy specimen taken from the injected lesion. These results

have formed the basis for an additional, larger clinical trial that is designed to assess the clinical activity of Ad5CMV-*p53* in combination with both chemotherapy and radiotherapy in newly diagnosed NSCLC patients.

The status of the various trials that have been conducted or that are ongoing with Ad5CMV-*p53* is indicated in Table 1. Ad5CMV-p53 in being developed by Introgen Therapeutics and Aventis Pharma.

d. Directions for the future

The apparent safety of Ad5CMV-*p53* in combination with standard cancer treatments such as surgery, radiotherapy, and chemotherapy supports its testing in combination approaches for many types of cancer where improved local/regional control will provide patient benefit. As has been seen in preclinical studies, the vector may act as a chemosensitizing or radiosensitizing agent that will increase the anti-tumor effects of these standard approaches. As an adjuvant to surgery, Ad5CMV-*p53* may act to eliminate microscopic disease that is left behind during a surgical resection. For patients who have disease that is resistant to standard approaches, it may act as a stand-alone agent whose anti-tumor effects provide patient benefit.

These approaches are being incorporated into the larger clinical trials that are designed to demonstrate the clinical benefit and safety necessary for approval of the product by the Food and Drug Administration and other worldwide regulatory authorities. These pivotal programs, usually designated as Phase III trials, are typically randomized, controlled studies where treatments that are standard for a particular type of cancer are used as the

control arm for the study. For one of the Phase III studies planned for Ad5CMV-*p53*, the experimental arm will be the vector administered as a single agent to refractory SCCHN patients who have failed multiple conventional treatments. The primary endpoint that will be evaluated will be patient survival. In another Phase III study in patients with recurrent, but not refractory, SCCHN, the experimental arm will be Ad5CMV-*p53* tested in combination with 5-fluorouracil and cisplatin, agents that are commonly used to treat this disease. The primary endpoint for this study will be the tine that it takes the tumor to progress. These studies are designed to form the basis for the first product approval for Ad5CMV-*p53*. Additional pivotal programs in other cancer types or with additional combinations may expand the indications for which Ad5CMV-*p53* will ultimately be used in standard clinical practice.

VI. GLOSSARY.

Oncogene – a gene that promotes the growth and survival of tumor cells. Oncogenes may derive from normal cellular genes and acquire altered growth promoting activity through mutation or deregulated (excessive) expression.

Tumor Suppressor – a gene that inhibits the growth and survival of cancer cells. Tumor suppressors are normal cellular genes but are frequently found to be missing in cancer cells.

p53 – a tumor suppressor gene encoding a cellular protein capable of inducing growth arrest and death of abnormal cells, including cancer cells. The p53 gene is often lost in cancer cells due to gene deletion or mutation.

Gene therapy –A therapeutic modality in which genes are transferred to a cell in order to alter the properties of the cell. Gene therapy is often accomplished by incorporating the gene of interest into a virus (vector) and then using the natural cellular entry mechanisms of the virus to achieve gene transfer. Tumor suppressor gene therapy transfers normal ("wild-type") forms of tumor suppressor genes such as p53 into the cancer cell to achieve growth arrest or cell death.

Wild-type – genetic term designating the normal form of the gene.

Transgenic mouse – A genetically engineered mouse carrying a known gene alteration. Such animals provide a means to evaluate the role of specific genes in normal development and in specific diseases such as cancer.

Adenovirus type 5 – A human DNA virus that has provided the basis of gene therapy vectors presently in clinical use.

Chemotherapy – A form of therapy that employs chemicals to achieve growth arrest or cell death of cancer cells.

Nude mouse – A strain of laboratory mice often used for preclinical studies of human cancer. Nude mice do not reject human cells as most mice do, and can therefore be used to study the response of human tumor cells (usually implanted under the skin of the mouse) to various treatments, prior to testing these treatments in clinical trials in humans.

Phase I clinical trial – The first step in clinical testing of a new treatment. A phase I trial is a controlled, clinical research study of the safety based on a small number of patients and conducted over the period of about a year.

Phase II clinical trial – The second step in clinical testing of a new treatment. A phase II trial is a controlled, clinical research study of the efficacy of a new treatment based on several hundred patient volunteers, generally conducted over a period of about two years.

Phase III clinical trial.- The final step of clinical of a new treatment A controlled, clinical research study of the efficacy of a new treatment based on a larger group of patient volunteers, generally conducted over a period of about three years in order to assess long term benefit.

VIII. BIBLIOGRAPHY.

Clayman, G.L., et al. (1998) Adenovirus-mediated p53 gene transfer in patients with advanced recurrent head and neck squamous cell carcinoma. J. Clin. Oncol. 16:2221-2232.

Donehower, L.A., et al. (1995) Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. Genes Dev.9:882-895.

Gjerset, R.A., et al. (1995) Use of wild-type p53 to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. Mol. Carcinog. 14:275-285.

Goodwin, W.J., et al. (1999) Randomized Phase II study of intratumoral injection of two dosing schedules using a replication-deficient adenovirus carrying the p53 gene (Ad5CMV-*p53*) in patients with recurrent/refractory head and neck cancer. J. Clin. Oncol. 18:1717A.

Kubba, S., et al. (2000) Phase I trial of adenovirus p53 in bronchioloalveolar cell lung carcinoma (BAC) administered by bronchoalveolar lavage. J. Clin. Oncol. 19:1904A.

Lang, F.F., et al. (2000) Preliminary results of a Phase I clinical trial of adenovirus-mediated p53 gene therapy for recurrent gliomas: biological studies. J. Clin. Oncol. 19:1785A.

Levine, A.J. (1993) The tumor suppressor genes. Annual Rev. Biochem. 62:623-651.

Logothetis, C.J., et al. (1999) Ad5CMV-p53 intraprostatic gene therapy preceding radcal prostatectomy (RP): an *in vivo* model for targeted therapy development. J. Clin. Oncol. 18:1203A.

Lotem, J., et al. (1993) Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents. Blood 82:1092-1096.

Lowe, S.W., et al. (1993) p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74:957-967.

Nemunaitis, J., et al. (2000) Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. J. Clin. Oncol. 18:609-622.

Nguyen, D.M., et al. (1996) Gene therapy for lung cancer: enhancement of tumor suppression by a combination of sequential systemic cisplatin and adenovirus-mediated p53 gene transfer. J. Thorac. Cardiovasc. Surg. 112:1372-1376.

O'Connor, P.M., et al. (1997) Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. Cancer Res. 57:4285-4300.

Pagliaro, LC. (2000) Gene therapy for bladder cancer. World J. Urol. 18:148-151.

Sweeney, P., et al. (2000) Ad5CMVp53 gene therapy for locally advanced prostate cancer--where do we stand? World J. Urol. 18:121-124.

Swisher, S., et al. (2000) A Phase II trial of adenoviral-mediated p53 gene transfer (RPR/INGN 201) in conjunction with radiation therapy in patients with localized non-small cell lung cancer (NSCLC). J. Clin. Oncol. 19:1807A.

Swisher, S.G., et al. (1999) Adenovirus-mediated p53 gene transfer in advanced non-small-cell lung cancer, J. Natl. Cancer Inst. 91:763-771.

Wolf, J.K., et al.(2000) A Phase I trial of Adp53 for ovarian cancer patients: correlation with p53 and anti-adenovirus AB status. J. Clin. Oncol. 19: 1510A.

FIGURE LEGENDS

Figure 1. Viability of T47D breast cancer cells in vitro 6 days following treatment with Adp53 or Adßgal (control) and 5 days following a 1 hour treatment with the indicated doses of cisplatin. Each curve is normalized to its own zero (virus only, no cisplatin), which for the Ad5CMV-*p53* (Adp53)-treated cells was 60% of the Adßgal treated cells.

Figure 2. Suppression of established subcutaneous tumors of FaDu head and neck cancer cells in nude mice by various treatments. The cisplatin-treated animals received cisplatin on days 1 and 15. The virus-treated animals received either Ad5CMV-*p53* (Adp53) or Adluc (control) on days 3,6,8, and again on days 17,20,22.

Table I.

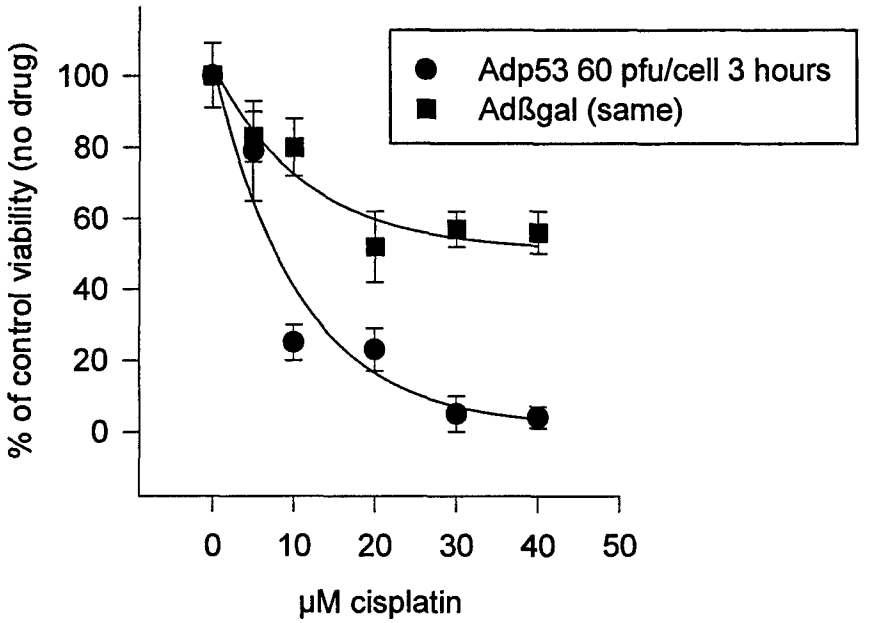
Development Status of Ad5CMV-*p53* (RPR/INGN 201)

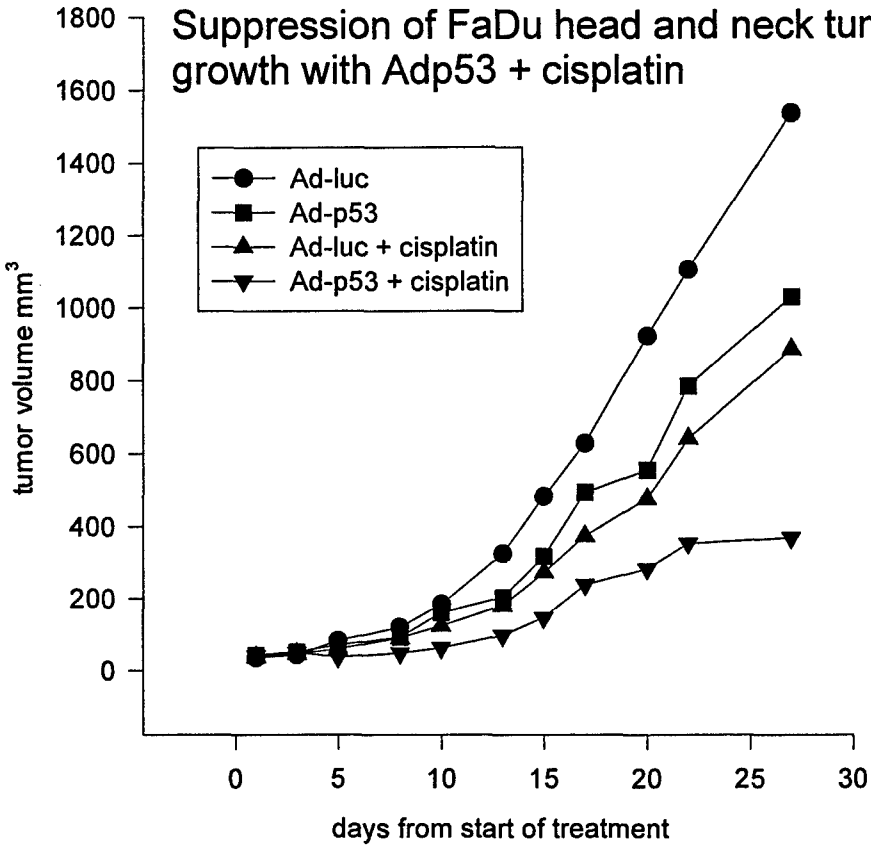
Indication	Development Phase	Combination Study?	Status*
Squamous cell carcinoma of the head and neck (SCCHN)	Phase III	Yes, with chemotherapy	Not yet enrolling patients
SCCHN	Phase III	No	Ongoing
SCCHN	Phase II	No	Completed
Non-small cell lung cancer (NSCLC)	Phase II	Yes, with radiotherapy	Ongoing
Prostate cancer	Phase I	No	Completed
Brain cancer**	Phase I	No	Ongoing
Ovarian cancer**	Phase I	No	Ongoing
Bladder Cancer**	Phase I	No	Ongoing
Breast cancer**	Phase I	No	Ongoing
Bronchoalveolar carcinoma**	Phase I	No	Ongoing
NSCLC	Phase I	Yes, with chemotherapy	Completed
SCCHN	Phase I	Yes, with surgery	Completed

* As of September 2000

** Conducted in conjunction with the National Cancer Institute.

In vitro suppression of T47D breast cancer cell viability by Adp53 and cisplatin




Suppression of FaDu head and neck tumor growth with Adp53 + cisplatin
Ad-luc
Ad-p53
Ad-luc + cisplatin
Ad-p53 + cisplatin
tumor volume mm3
1800
1600
1400
1200
1000
800
600
400
200
0
0
5
10
15
20
25
30
days from start of treatment

24

SENSITIZATION OF TUMORS TO CHEMOTHERAPY THROUGH GENE THERAPY

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1. INTRODUCTION

The cellular response to DNA damage plays a critical role not only in tumor progression but also in the process of acquired drug resistance, a problem that affects about half of all cancer cases overall and remains one of the major obstacles to successful therapy of cancer. Modulation of these DNA damage response pathways may therefore provide us with a means to reverse acquired drug resistance and improve the outcome of therapy for a large fraction of cancer patients. In this article we will focus on two major pathways involved in the cellular response to DNA damage: The Jun kinase stress activated pathway, and the p53-mediated DNA damage response pathway leading to apoptosis. Through independent mechanisms, each of these pathways modulates the cellular response to DNA damaging chemotherapies and radiation. Therapeutic approaches based on inhibiting the Jun kinase pathway and/or restoring the p53 pathway may, therefore, provide us with new biological strategies for reversing acquired drug resistance, thus improving the outcome of therapy for most cancers.

2. THE p53 TUMOR SUPPRESSOR AND THE DNA DAMAGE RESPONSE

Over half of all cancers suffer loss of function of the p53 tumor suppressor (Levine, 1993), a key player in the induction of apoptosis in response to DNA damage (Clarke *et al.*, 1993; Gjerset *et al.*, 1995; Lotem *et al.*, 1993; Lowe *et al.*, 1993), in addition to its roles in cell cycle regulation and DNA repair (Levine, 1997). Its involvement in DNA damage-induced apoptosis may derive from its ability to bind, alone or possibly in combination with other DNA damage recognition proteins, to sites of damaged DNA, including single stranded ends and insertion-deletion loops (Bakalkin *et al.*, 1995; Lee *et al.*, 1995; Levine 1997 (review)). p53 might also bind to DNA adducts and strand breaks induced by various therapies. The frequent loss of p53 function in

Cancer Gene Therapy: Past Achievements and Future Challenges, edited by Habib
Kluwer Academic/Plenum Publishers, New York, 2000.

cancer, often associated with disease progression and increased genomic instability, may reflect at least in part the role of p53 in DNA damage recognition and apoptosis. Most genome destabilizing events, including gene amplification, gene deletion and gene translocation, involve DNA strand breaks (Stark, 1993). These breaks could serve as triggers for p53-mediated apoptosis and provide the driving force for loss of p53.

The same process that underlies the progression of cancer, that is, genomic instability accompanied by loss of p53-mediated apoptosis, can also lead to therapy resistance. Support for the idea that loss of p53 could desensitize a cell to the damaging effects of drugs and radiation comes from studies of p53-null transgenic mice. In these studies it was observed that normal transgenic hematopoietic cells (Lotem and Sachs, 1993), E1A-expressing trangenic fibroblasts (Lowe *et al.*, 1993), and transformed transgenic fibroblasts (Lowe *et al.*, 1994) were all more resistant to apoptosis following treatment with any of a wide variety of anti-cancer agents including radiation, than were the comparable cells from the parental strain of mice that express wild-type p53. Cell killing was therefore enhanced in cells that expressed wild-type p53 and were able to trigger their own cell death program.

The possibility that p53 status is a factor in therapy responsiveness finds further support from a recent anti-cancer drug screening of human tumor cell lines expressing mutant or wild-type p53 (O'Connor *et al.*, 1997). In that study the growth inhibitory properties of some 123 anti-cancer agents were tested in 60 human tumor lines of known p53 status. It was found that cells expressing mutant p53 showed less growth inhibition than did wild-type p53-expressing cells lines following treatment with the majority of clinically used anti-cancer agents, including DNA cross-linking agents such as cisplatin, antimetabolites such as 5-fluorouracil, and topoisomerase I and II inhibitors. Since these agents are known to cause DNA damage, either directly or indirectly, these results are consistent with a role of p53 in mediating the cellular apoptotic response to DNA damage. One class of agents, the antimitotic agents, appeared to suppress growth in a p53-independent manner, consistent with the primary target for these agents being the mitotic apparatus rather than DNA.

Taken together, these studies suggest that p53 gene transfer could have clinical application in suppressing cancer and enhancing the responsiveness of tumors to a wide variety of DNA damaging therapies, a possibility that greatly expands the clinical application of p53-based approaches. Numerous *in vitro* studies in a variety of tumor cell systems support the use of p53 gene transfer to sensitize tumors to such therapy, including, 5-fluorouracil, cisplatin, topoisomerase I inhibitors and gamma radiation (see below).

2.1. *In Vitro* Sensitization of Tumor Cells to Chemotherapeutic Drugs by p53

In Figs. 1 and 2 we have summarized the results of screening tumor cells for sensitivity to various chemotherapeutic agents following exposure of cells to a replication-defective adenovirus encoding wild-type p53 (Ad-p53, Ad-βgal, and Ad-Luc (luciferase) vectors provided by Dr. Deborah R. Wilson, Introgen Therapeutics, Inc.). The cells were treated with virus under conditions which achieve about 70–80% infection efficiency, as judged by infection of parallel cultures with a β-galactosidase adenovirus. Following infection, cells were replated at low density in 96-well plates and treated with varying levels of chemotherapeutic agent, followed by an additional 5–7 days of incubation and measurement of cell viability.

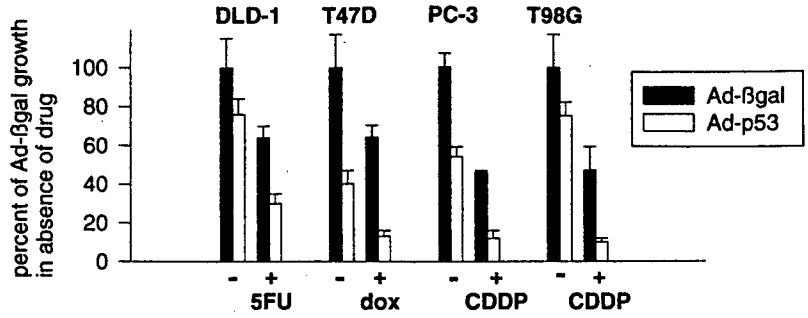

Figure 1. Viability assay showing that Ad-p53 suppresses growth and enhances sensitivity to DNA damaging chemotherapeutic drugs in p53-mutant-expressing cells (DLD-1 colon cancer, T47D breast cancer, PC-3 prostate cancer, and T98G glioblastoma). Infection efficiencies were 60–70% and drug treatments 1 day post-infection were as follows: 5-fluorouracil (5FU) 10 μM 1 hour; doxorubicin (dox) 3.7 μM 1 hour; cisplatin (CDDP) 30 μM 1 hour for PC-3 and 20 μM 1 hour for T98G. Viability was assayed 6 days post drug treatment in all cases except PC-3 which was 4 days post drug treatment, and expressed as a percent of control cells treated with Ad-βgal.

In numerous examples from a variety of cancer types, we have observed suppression of mutant-p53-expressing tumor cells *in vitro* following treatment with Ad-p53. As shown in Fig. 1, restoration of wild-type p53 in mutant p53-expressing cells (DLD-1, T47D, PC-3, T98G) results in marked enhancement of sensitivity to a variety of DNA damaging treatments (5-fluorouracil, doxorubicin, cisplatin), consistent with possibility that we have restored the p53-mediated pathway of DNA damage recognition and apoptosis. Apoptosis was confirmed by (a) propidium iodide staining of fixed cells followed by FACS analysis to reveal a sub G1 peak of apoptotic cells, and (b) an ELISA assay (Boehringer Mannheim Corp, Indianapolis, IN)) to detect oligonucleosomal fragments released from the nuclei of cells in the early phases of apoptosis (see Gjerset *et al.*, 1995).

In contrast, this enhancement of sensitivity is not observed in two wild-type p53-expressing cell lines, MCF7 and LS174T (Fig. 2). This suggests that wild-type p53 gene transfer may be effective in therapy sensitization only in the case of tumors that have lost wild-type p53 function.

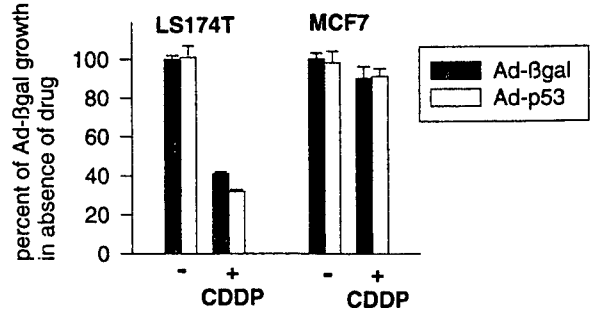

Figure 2. Viability assay showing that Ad-p53 neither suppresses growth nor enhances sensitivity to DNA damaging chemotherapeutic drugs in wild-type p53-expressing cells (LS174T colon cancer cells and MCF7 breast cancer cells). Infection efficiencies were 60–70%, and drug treatments 1 day post-infection were 30 μM cisplatin (CDDP) 1 hour for LS174T and 20 μM 1 hour for MCF7. Viability was assayed 6 days post drug treatment.

2.2. *In Vivo* Sensitization of Tumors to Chemotherapeutic Drugs by p53

We have extended our *in vitro* observations on tumor suppression and therapy sensitization to *in vivo* models for human head and neck cancer and colon cancer (Gjerset *et al.*, 1997) and prostate cancer (Fig. 3) in nude mice, where established tumors were treated *in vivo* with replication-defective p53-adenovirus, and chemotherapy. We have also studied chemosensitization by p53 using *ex vivo* modified cells in an orthotopic model of glioblastoma in Fisher rats (Dorigo *et al.*, 1998). The results are consistent with other *in vivo* studies in animal models showing a combined benefit of p53 and chemotherapy (Badie *et al.*, 1998; Fujiwara *et al.*, 1994; Miyake *et al.*, 1998; Nielsen *et al.*, 1998; Nguyen *et al.*, 1996). Together with the *in vitro* data, these results support the clinical application of adenovirus p53 combination approaches to tumors expressing mutant p53. In the experiment shown in Fig. 3, PC-3 prostate cancer cells which express mutant p53, were implanted in 20 nude mice (5×10^6 cells/animal) where they form rapidly growing subcutaneous tumors. At 5 days post-implantation, when tumor sizes had obtained a volume of about $50\,mm^3$, animals were randomized by tumor size and treatment was initiated. The first day of treatment was then designated day 1 and consisted of intraperitoneal injection of cisplatin (Platinol™, Bristol Laboratories, obtained through local pharmacies), at 4 mg/kg (LD10) on days 1 and 8. Vector (Ad-p53 or Ad-luc control) was administered intratumorally (10^8 pfu per tumor) of days 1, 3, 5, 8, and 10. In both cases, some suppression was observed with Ad-p53 alone, with a significant enhancement of suppression when Ad-p53 was combined with chemotherapy. The vector doses used here

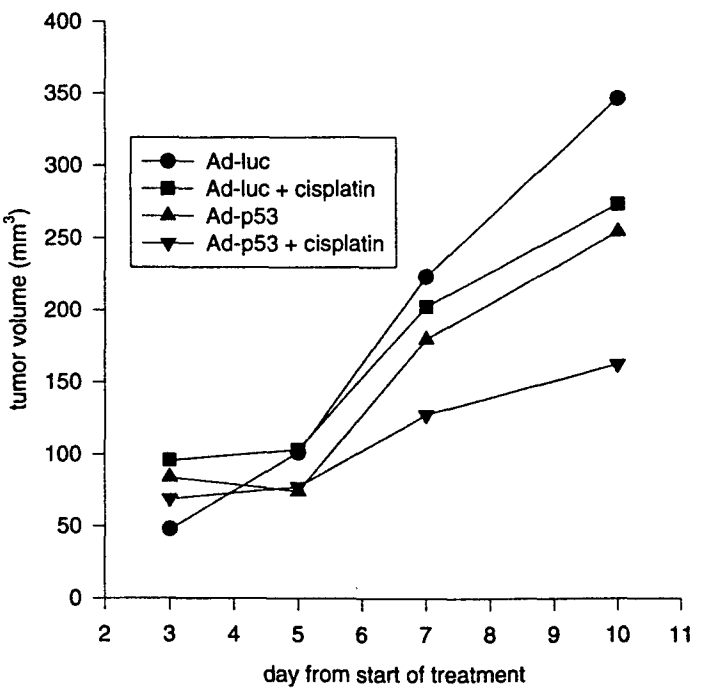

Figure 3. Suppression of PC-3 prostate tumor growth in nude mice by Ad-p53 plus cisplatin. Vector (10^8 pfu per tumor) was administered on days 1,3,5,8,10, and cisplatin was administered on days 1, 8.

were relatively low (10–30 pfu per tumor cell) and completely without side effects (as judged by periodic weight measurements on the animals and histological examination. We observe little or no toxicity in nude mice of vector doses as high as 10^9 pfu per animal (Gjerset *et al.*, 1997). Phase I clinical trials employing p53 adenovirus also demonstrate that this vector is well tolerated in patients (Roth *et al.*, 1998). Thus restoration of wild-type p53 function through gene therapy may provide a significant benefit for patients with advanced cancers, when used in combination with conventional therapies.

3. THE JUN KINASE/STRESS-ACTIVATED PROTEIN KINASE PATHWAY AND THE DNA DAMAGE RESPONSE

Another important cellular pathway is also triggered in response to DNA damage: the Jun Kinase/Stress-activated protein kinase pathway (JNK/SAPK), one of several distinct Mitogen-Activated Protein Kinase (MAPK) Pathways involved in signal transduction. Besides its role in the DNA damage response (discussed below), the JNK/SAPK pathway is also induced by growth factors such as EGF-1(Bost *et al.*, 1999), by oncogene expression (Binétruy *et al.*, 1991), and is essential for transformation of rat embryo fibroblasts (Smeal *et al.*, 1991). As we show below, inhibition of this pathway enhances sensitivity to DNA damaging therapies in both p53 mutant and p53 wild-type tumors, as T98G glioblastoma cells, PC-3 prostate cancer cells, and U87 glioblastoma cells which express mutant p53, and MCF7 breast cancer cells which express wild-type p53, are all sensitized to various DNA damaging treatments by expression of a dominant-negative inhibitor of the pathway. Thus it may be possible to modulate this pathway by itself or in combination with p53 gene replacement to enhance tumor cell responsiveness to DNA damaging chemotherapies.

The Mitogen Activated Protein Kinase (MAPK) pathways play roles in several cellular processes, including cellular transformation, proliferation, differentiation, and the DNA damage response. Through these pathways extracellular growth and stress stimuli transmit signals through a cascade of kinases and phosphorylation events that result in the phosphorylation and activation of transcription factors such as c-Jun (a heterodimeric component of the AP-1 complex and related transcription complexes), ATF-2 and Elk-1 (Bost *et al.*, 1997; Cavigelli *et al.*, 1995; Dérijard *et al.*, 1994; Gupta *et al.*, 1995; Hibi *et al.*, 1993; Livingstone *et al.*, 1995; Potapova *et al.*, 1997; Smeal *et al.*, 1991, 1992). This phosphorylation activates the transcriptional transactivation properties of AP-1 and related factors and leads to the subsequent induction of specific response genes. The cascade of events begins with the activation of the Mitogen Activated Protein Kinase Kinase Kinases (MAPKKK), followed by activation of the Mitogen Activated Protein Kinase Kinases (MAPKK), and finally activation of the Mitogen Activated Protein Kinases (MAPK), of which the Jun Kinase family of enzymes (including JNK 1,2,3) is one example.

3.1. *In Vitro* Sensitization to Chemotherapeutic Drugs Through Inhibition of the JNK/SAPK Pathway

T98G glioblastoma cells activate the JNK/SAPK pathway in response to treatment with the chemotherapeutic agent, cisplatin (Potapova *et al.*, 1997), which forms bifunctional DNA cross links between adjacent guanines or adenine-guanine dinucleotides. This

is consistent with numerous other reports in which activation of the JNK/SAPK pathway has been observed in response to genotoxic DNA treatments, including UV irradiation (Dérijard *et al.*, 1994, Adler *et al.*, 1995a, 1995b, 1996), ionizing radiation (Kharbanda *et al.*, 1995), the mutagens methylmethane sulfate (MMS), N-nitro-N′-nitroso-guanidine (MNNG) and numerous genotoxic chemotherapeutic agents such as Ara-C (1-β-D-Arabinofuranosylcytosine) (van Dam *et al.*, 1995; Kharbanda *et al.*, 1995). For several of these agents such a UV-irradiation, the activation of the JNK/SAPK pathway is directly proportional to the number of DNA damaging events (Adler *et al.*, 1995a). Activation is often rapid, detectable within a few minutes. These characteristics have given rise to the hypothesis that the role of the JNK/SAPK pathway may be to mediate DNA repair (Potapova *et al.*, 1997).

To evaluate the role of JNK/SAPK activation in the cellular DNA damage response we selected clones of T98G cells modified to express a non-phosphorylatable mutant of c-Jun (T98G mJun), in which serines 63 and 73, the targets of Jun Kinase-mediated phosphorylation, have been replaced by alanines (Smeal *et al.*, 1991, 1992). These cells are therefore suppressed in JNK-mediated functions. We found that unlike control vector-modified cells (T98GLHCX) or wild-type c-Jun-modified cells (T98GcJun), both of which are highly resistant to cisplatin, the modified clones expressing mJun (T98GdnJun) showed a significant loss of viability following treatment with cisplatin (Fig. 4). Furthermore, the increase in cisplatin sensitivity correlated with the level of expression of mJun in several different clones (Fig. 5). We observed a similar increase in sensitivity to cisplatin in MCF7 breast cancer cells, in PC-3 prostate cancer cells, and in U87

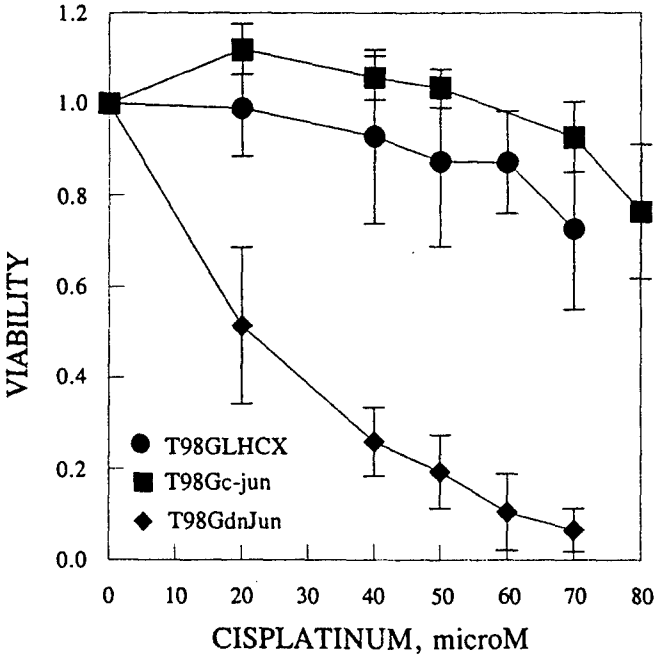

Figure 4. Viability assay showing that mutant Jun sensitizes T98G cells to cisplatin. Viability was assayed 5 days following a 1 hour treatment with cisplatin. Empty vector control cells (●); wild-type c-Jun-expressing cells (■); and mutant Jun-expressing cells (◆).

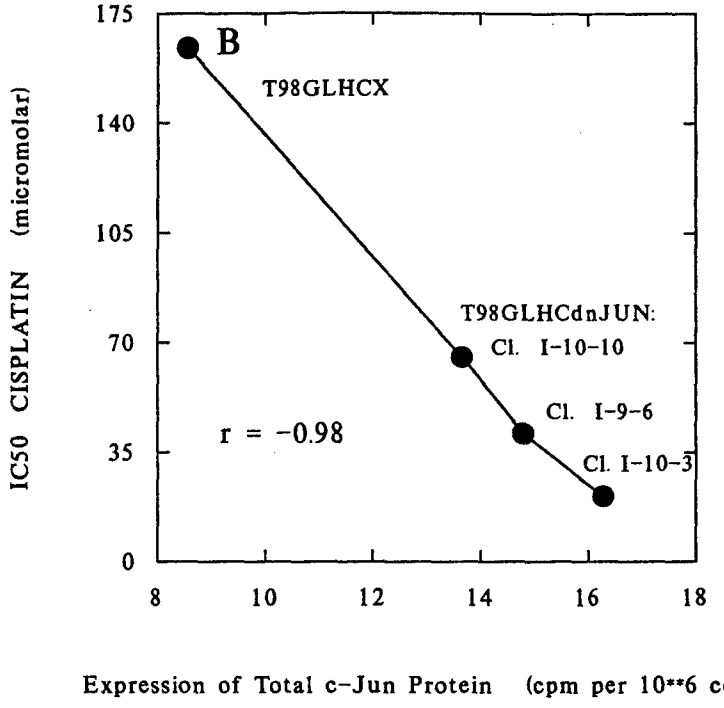

Figure 5. Dose response curve of the IC_{50} (cisplatin) versus total immunoreactive Jun (c-Jun + mutant Jun). Total immunoreactive Jun was determined by sequential immunoprecipitation of ^{35}S-labeled cells using specific Jun B, Jun D, followed by pan-Jun antiserum.

glioblastoma cells that were modified with the non-phosphorylatable mJun (not shown). We also observed enhanced sensitivity to cisplatin in cells modified with the TAM67 mutant of c-Jun (not shown). Tam67 has a truncated N-terminal domain and is known to be a dominant negative inhibitor of c-Jun phosphorylation (Grant *et al.*, 1996). However, we did not observe in mJun modified T98G cells nor in mJun modified PC-3 cells an increase in sensitivity to the anti-mitotic agent, taxotere™ (kindly provided by Dr. Pierre Potier, Centre Nationale de la Recherche Scientifique, Paris), whose primary target is the mitotic spindle rather than DNA (Fig. 6). Taken together these results argue that the JNK/SAPK pathway plays a role in resistance to DNA damaging agents but not to agents that do not damage DNA, and that inhibition of this pathway could be a means to reverse resistance to DNA damaging therapies used in cancer treatment.

3.1.1. Inhibition of DNA Repair by Inhibition of the JNK/SAPK Pathway. T98G clones modified to express mutant Jun are compromised in repair of cisplatin-DNA adducts (Potapova *et al.*, 1997), an observation that further supports the rationale for enhancing sensitivity to DNA damage through inhibition of the JNK/SAPK pathway. DNA repair is known to play a role in acquired resistance to DNA damaging chemotherapies (Barret and Hill, 1998; Crul *et al.*, 1997; Reed, 1998; Fink, Aebi and Howell, 1998; Saves and Masson, 1998; Scanlon, 1989). Furthermore, as summarized in Table 1, a number of the genes involved in DNA synthesis and repair are potentially regulated by the AP-1 transcription factor and related transcription factors targeted by the

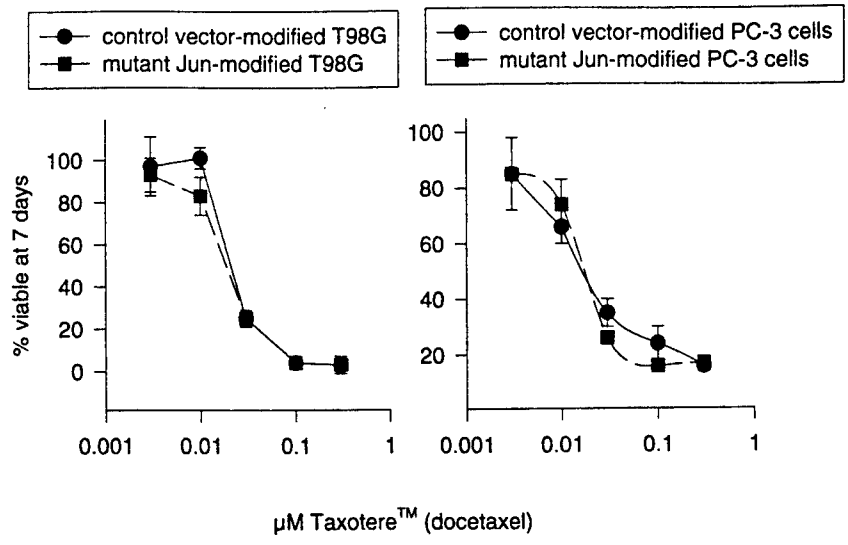

Figure 6. Viability assay showing that mutant Jun does not sensitize T98G cells to Taxotere™. Viability was assayed 7 days following a 1 hour treatment with Taxotere™.

JNK/SAPK pathway (i.e., c-Jun and ATF2, components of AP-1 and c-Jun-ATF2 heterodimeric transcription activating complexes, respectively). Furthermore, down regulation of AP-1 by treatment of cells with a c-fos antisense ribozyme, has previously been shown to correlate with down regulation of expression of certain of these enzymes (thymidylate synthetase, DNA polymerase β) and enhanced sensitivity to cisplatin (Scanlon *et al.*, 1991).

We analyzed DNA repair using a PCR-based assay developed by Jennerwein and Eastman (1991) based on their observations that DNA-cisplatin adducts block the progression of the Taq polymerase and lead to a decrease the yield of PCR product obtained from any given PCR amplicon in proportion to the extent of platination. The authors demonstrated that the relationship between the relative PCR signal strength (P) for a given amplicon from damaged versus undamaged templates, and overall platination level fits a Poisson distribution predicted from a random platination, so that $P = e^{-n}$, where n = adducts per amplicon-sized fragment.

We have used primers described by Oshita and Saijo (1994) to amplify a 2.7 Kb fragment of the human hypoxanthine phosphoribosyl transferase (HPRT) gene, a fragment large enough to sustain readily detectable levels of damage following cisplatin treatment of cells. As an internal control for the efficiency of the PCR reaction, we used a nested 5′ primer which amplified a 150 base fragment of the same gene. At levels of cisplatin used to treat cells, damage to the smaller fragment was undetectable. PCR reactions were performed under quantitative conditions such that the extent of reaction remained directly proportional to amount of template. Figure 7 shows an example of PCR amplification of the 2.7 Kb fragment and the 150 base fragment from DNA prepared from T98G glioblastoma cells treated with 0, 100, and 200 μM cisplatin for 1 hour. Quantitation of band intensities was accomplished using Kodak digital camera and analysis software, and relative band intensity measurements were then used to calculate adducts per Kb based on the Poisson relationship described above.

Table 1. DNA REPAIR ASSOCIATED GENES WHICH CONTAIN POTENTIAL JNK-REGULATED SEQUENCES[1]

Repair-associated gene	Element	Sequence (consensus sequence)[2]	Position[3]	LLG score	Reference for function
DNA Polymerase β	AP-1/TRE	CTGACTCA (-tgactca)	337	2.0	Known to be functional and TPA-activated classic TRE (1).
	ATF/CREB	TTACGTAA (ttacgtca)	282	2.0	Known to be genotoxic-activated (1, 9).
DNA Polymerase α (26)	ATF/CREB	CACGTCA (t/gacgtca)	−82		Function of ATF/CREB (26) unknown.
		GGGGTCA (tgagtca)	−149		AP-1 sites thought to be significant in DNA repair: DPα is over-expressed in cisplatin resistent cells and anti-Fos ribozyme sensitizes (27).
Topoisomerase I	AP-1	GGGGGCGG (tgagtca)	753	2.0	(2–3)
	AP-1	TGACCCA (tgactca)	217	2.0	(2–3)
	ATF/CREB	TGACGTCA (tgacgtca)	792	2.0	known to be functional & stress-activated (2–3).
Topoisomerase IIα	ATF/CREB	TGACGCCG (tgacgtca)	286	2.0	(10–11); Topo II is UV-inducible and functions early in UV-induced DNA damage repair.
	AP-1	TGATTGG (tgagtca)	337	2.0	(10)
Topoisomerase IIβ				2.0	(12)
	AP-1	TGACTCA (tgactca)	3	1.6	(12)
	AP-1	AGAGTCA (tgagtca)	65		
ATF-3	AP-1	TGAGTAA (tgacgtca)	−1,600		(13) ATF-3 is stress-induced, anisomycin (JNK activator) induced, and induced by ATF-2/c-Jun coexpression suggesting a functional role for the ATF/CREB site. ATF-3/c-Jun heterodimers bind ATF/CREB sites and activate transcription (14–15) and ATF-3/c-Jun and ATF-3/JunD heterodimers have been shown to bind TTAGTTAC, a ATF/CREB sequence, which mediates EGF/ras/raf-stimulated transcription (28), however, a role in induction of DNA repair genes is not known.
	AP-1	ATAGTCA (tgacgtca)	−1,353		
	AP-1	AGACTAA (tgacgtca)	−605		
	AP-1	GAGTCA (tgacgtca)	−380		
	ATF/CRE	TTACGTCA–92 (ttacgtca)			

(*continued*)

Table 1. ***Continued***

Repair-associated gene	Element	Sequence (consensus sequence)[2]	Position[3]	LLG score	Reference for function
c-Jun	ATF/CREB	TTACCTCA (t t a c g t c a)		2.0	(4–5); the "functional" association with DNA repair is strong induction of c-*Jun* by genotoxins known to activate JNK/SAPK
Uracil Glycosylase	AP-1	TGGGTCA (t g a g t c a)	141	2.0	not known
PCNA	AP-1	TGACTCA (t g a c t c a)	489	2.0	DNA polymerase-•• accessory protein function;
Proliferating Cell Nuclear Antigen	ATF/CREB	TGAGGTCAGGG (t g a c g t c a - - -)	209	1.64	(7–8); Il-2, a potent JNK/SAPK activator, induces PNCA expression via ATF/CREB promoter sites which is blocked by rapamycin.
	ATF/CREB	GTGACGTCAC (- t t a c g t c a - -)	1,253	1.60	
GADD153 (19)	ATF/CREB	ACTCCTGACCTT (t g/t a c g t c a - - - -)	207	1.63	Induction requires phosphorylation-dependent event that is *not* PKA, PKC (16), or p38 (25) mediated consistent with a role for JNK/SAPK (16). Moreover GADD153 is induced by MMS (16) & cisplatin (17–18). The role of the ATF/CREB site is unknown. GADD153 is phoshorylated and activated by p38 in response to stress (25).
Growth Arrest and DNA-damage" Inducible Gene	AP-1	TGACTCA (t g a c t c a)	710	2.0	
XRCC1 (20)	ATF/CREB	ACGTCA (a c g t c a)	1,815	2.0	The site at 466 is consistent with c-Jun/ATF-3 *vs.* ATF-2 (TESS).
X-ray Damage Repair Cross Completementing Gene Product.	ATF/CREB	GGACGTCAA (t g a c g t c a)	1,814	2.0	Functional roles of these ATF/CREB and AP-1 sites are not known zxzx.
	ATF/CREB	CCTGACCTCA (- - t g a c g t c a)	2,029	1.64	
	ATF/CREB	GCTGACGTCAG (- - t g a c g t c a -)	466	1.60	
		CCAATCA (t g/t a c/g t c a)	93	2.0	

MGMT (22)	ATF/CREB	TGCGTCA (t g a c g t c a)	1,661	2.0	MGMT is induced by genotoxic agents (21). The site at 1,674 is consistent with c-Jun/ATF-3 (TESS). The functional significance of these sites is unknown.
O6-Methylguanine-DNA-Methyl-transferase	ATF/CREB	GTGACATCAT (- t g a c t c a -)	1,195		
	AP-1	TGAGTCA (t g a g t c a)	734	2.0	
	AP-1	TTACTCA (t t a c t c a)	285	1.73	
MSH2 (23)	ATF/CREB	TGGCGTCA (t g a c t c a)	108	1.62	TESS does not recognize c-Jun participation at 108 site. Role in cisplatin induced repair unknown. MSH2 has been reported to selectively bind to cisplatin-DNA adducts
	AP-1	TGAATCA (t g a c/g t c a)	569	2.0	
	AP-1	TGATGAAA (t g a c/g t c a)	884	1.62	
Metallothionein IIA	AP-1	GAGCCGCAAGT (g a g t c a - - - - t GACTTCTAGCG g a c t c a a g t c CGGGGCGTG a - - - - - - t g)	188	2.0	(6); TPA and UV-light activated

[1]Repair associated protein for which only partial promoter sequences are known (i.e. in Genbank) without recognizable AP-1 regulated sites include ADP ribose poly merase, tif2/ref1, SSRP, Ercc1 and thymidylate synthetase.

[2]AP-1 consensus: TG/TAC/GTCA; CREB/ATF-2 consensus: TG/TACGTCA.

[3]Positions are based on TESS numbering of promoter sequences unless preceeded by (–).

References: 1. Srivastava *et al.* J. Biol. Chem., 1995;270:16408. 2. Baumgärtner *et al.*, Biochem. Biophys. Acta, 1994;1218:123. 3. Heiland *et al.* Eur. J. Biochem. 1993;217:813. 4. Kharbanda *et al.* Canc. Res., 1991;51:6636. 5. Kharbanda *et al.*, J. Clin. Inves., 1990;86:1517. 6. Lee *et al.* Nature, 1987;325:368. 7. Feuerstein N. *et al.* J. Biol. Chem., 1995;270:9454. 8. Huang D. *et al.* Molec. Cell. Biol., 1994;14:4233; 9. Kedar *et al.* PNAS USA, 88:3729. 10. Hochhauser D. *et al.* J. Biol. Chem., 1992;26:187961–187965. 11. Popanda O. Thielmann H.W. Carcinogenesis, 1992;13:2321. 12. Unpublished: Genebank Acc. #T29334, Inst. Genomics Res., 1995. 13. Liang *et al.* J. Biol. Chem., 1996;271:1695. 14. Hsu, J.-C. *et al.*, Mol. Cell. Biol., 1992;12:4654. 15. Chu, H.-M. *et al.* Mol. Endocrinol. 1994;8:59. 16. Luethy, J. and Holbrook, N. Cancer Res. 1994;54:1902S. 17. Gately, D. *et al.* Br. J. Cancer, 1994;70:1102. 18. Delmastro, D. *et al.* Canc. Chemoth. Pharmacol., 1997;39:245. 19. Park, J. *et al.*, Gene, 1992;116:259 (Acc. #S40707). 20. Lamerdin, J., *et al.*, Genomics, 1995;25:547 (Acc. #L34079). 21. Lefebvre, P. *et al., Dna Cell Biol.*, 1993;12:233. 22. Iwakuma, T. *et al.*, DNA Cell Biol., 1996;15:863. 23. Seib, T. *et al.* Gene Bank direct submission Acc. #U232824. 24. Leach, F. *et al.* Cell, 1993;75:1215. 24. Kolodner, R. *et al.* Genomics, 1994;24:515. 24. Mello, J. *et al.* Chem. Biol. 1996;3:579. 25. XiaoZhong, W. and Ron, D. Science, 1997;272;1347–1349. 26. Pearson, B. *et al.* Molec. Cell. Biol., 1991;11:2081. 27. Scanlon, K. *et al.* PNAS USA, 1994;91:11123. 28. Nilsson *et al. Cell Growth & Diff.* 1997;8:913.

Abbreviations: LLG, Log likeli-hood score which is 2 for a perfect match of the candidate response element with with the consensus sequence (TESS criteria) and all ambiguous matches yield a score of 0; AP-1, activator protein-1 complex, a Jun and a Fos family member; CREB/ATF, cAMP response element binding proteins, N-terminal phosphorylated c-Jun and N-terminal phosphorylated ATF-2 for a cAMP-independent complex which bind the CRE-like sequence TTACGTCA. XRCC., MMS, methyl methanesulfonate.

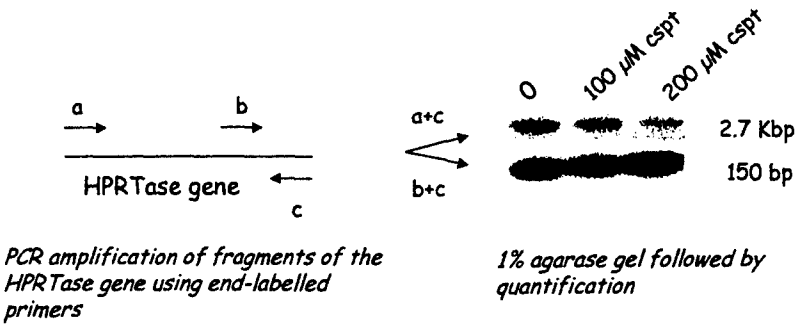

Figure 7. PCR amplification of DNA from T98G cells that had been incubated 1 hour with 0, 100 μM, and 200 μM cisplatin. Products were analyzed on an agarose gel and stained with ethidium bromide, followed by quantitation of band intensities using the Electrophoresis Documentation and analysis System 120 (Kodak Digital Science™).

Table 2 summarizes the cisplatin adducts per Kb observed on DNA from T98G glioblastoma cells treated for 1 hour with 200 μM cisplatin and then harvested either immediately or after a 6 hour recovery period. As shown in the table, cisplatin adducts occur at greater frequency in DNA from T98G mutant Jun expressing cells than in DNA from parental T98G cells. Furthermore, parental T98G cells repair more than half of the adducts during the 6 hour recovery period, whereas little repair occurs in T98G mJun cells during that period of time. Thus the increase in cisplatin sensitivity observed in T98G mJun cells can be accounted for at least in part by a defect in their ability to repair damaged DNA.

3.2. Mechanism of Action of JNK/SAPK in DNA Repair

How might the activation of JNK by genotoxic stress affect DNA repair? The answer to this question is not known in any detail, but a number of observations point to several testable hypotheses. It was noted that the promoters of certain genes to be involved in carrying out cisplatin-DNA adduct repair contain AP-1-like or ATF2/CREB regulatory elements (section 3.1.1). The repair of DNA-cisplatin adducts is believed to require the nucleotide-excision repair process (Reed, 1998). In addition, a number of enzymes utilized for DNA synthesis are reported to be involved (reviewed in Zamble and Lippard, 1995). These included DNA polymerase β, topoisomerase I, topoisomerase II, uracyl glycosylase, PCNA, metallothionine and others (Table 1, and references therein). In each case the promoter regions contains one or more AP-1

Table 2. Cisplatin-DNA adducts per 2.7 KBASE

Treatment	T98G parental cells	T98G mutant jun cells
200 μM cisplatin 1 hour	0.48 ± 0.08	0.63 ± 0.2
200 μM cisplatin 1 hour, 6 hour recovery	0.20 ± 0.05	0.55 ± 0.2

or ATF2/CREB regulatory sequences. In several cases such as DNA polymerase beta, genotoxic stress in the form of UV-irradiation is know to induce the gene and that the ATF2/CREB sites are required for this event (Table 1 and references therein). Moreover, the N-terminal phosphorylation of c-Jun and ATF2 by the action of Jun Kinase leads to increased DNA binding and transactivation potential of heterodimeric complex formed by phosphorylated c-Jun and ATF2. Indeed, the c-Jun promoter itself is one of the better studied example of a gene that is up-regulated upon binding of the c-Jun-ATF2 heterodimer (van Dam *et al.*, 1995; Wilhelm *et al.*, 1995). N-terminal phosphorylation of both c-Jun and ATF2 is required for the activation of the c-jun gene (van Dam *et al.*, 1995; Wilhelm *et al.*, 1995). Other examples of gene that are up-regulated upon formation of c-Jun-ATF2 heterodimers include the ELAN promoter (De Luca, *et al.*, 1994) and the TNF-alpha promoter (Newell *et al.*, 1994). Thus, there is circumstantial evidence supporting the hypothesis that activation of the Jun Kinase pathway by genotoxic stress leading to the phosphorylation of both ATF2 and c-Jun and thereby causing preferential formation of ATF2-c-Jun heterodimers. These complexes preferentially bind promoters containing octomeric ATF2/CREB regulatory elements. This hypothesis predicts that several of the genes known to be involved in cisplatin-DNA adduct repair may be coordinately upregulated upon activation of the JNK pathway following damage to DNA by cisplatin. If verified, such an explanation suggests that the JNK pathway is a potentially useful target for intervention and that agents that inhibit JNK activity or formation may be useful in achieving enhanced sensitivity of tumor cells to cisplatin.

3.3. JNK/SAPK Pathway with Antisense Approaches

The above observations argue that targeting the JNK/SAPK pathway could have potential as a sensitizer to a variety of DNA damaging chemotherapies, a possibility that we have investigated directly using antisense oligonucleotides to either JNK 1 or 2 family of JNK isoforms.

Antisense mechanisms result most likely from oligonucleotide-target mRNA hybrid formation in the nucleus which stimulates the cleavage of the mRNA at one or more sites near the terminus of the hybrid complex by ribonuclease H (Crooke, 1992, 1998; Dean *et al.*, 1996), as well as from cytoplasmic complexes which may lead to translation arrest (Crooke, 1992, 1998; Dean *et al.*, 1996). Because steady state mRNA levels do not always predict steady state gene product levels, we monitor the effects antisense by the fractional reduction of the gene product. An enzymatic assay that discriminates among the various isoforms of Jun Kinase can be used (Hibi *et al.*, 1993), as well as Western analysis or PAGE analysis following immunoprecipitation.

The appropriate oligonucleotide controls for antisense efficacy have been described in detail (Stein and Kreig, 1994). and include a sense sequence, scrambled sequence or random control oligonucleotides, and antisense sequences bearing one or a small number of mismatched bases, i.e. mismatched with respect to the complementary target sequence.

For our studies we have used antisense oligonucleotides complementary to the JNK1 or JNK 2 families of JNK isoforms by selecting target sequences common to the major isoforms of human JNK1 and JNK2. Phosphorothioate backbone chemistry has been used as this formulation is nuclease resistant, readily available and a common element of second generation compounds (Crooke, 1992, 1998; Dean *et al.*, 1996; McKay *et al.*, 1999).

In collaboration with ISIS Pharmaceuticals, Inc. (Carlsbad, CA) we have identified effective antisense oligonucleotides capable of entering and inhibiting specifically JNK1

or JNK2 mRNA expression in A549 lung cancer cells (Bost *et al.*, 1997, 1999). As an example of a screening for JNK1 and JNK2, Fig. 8 is included. As shown, most candidate oligonucleotides had only a small effect on the steady state level of JNK1 or JNK2 mRNA (Fig. 8). However, their degree of activity greatly varied depending on the targeted region (Fig. 8). Among the most potent oligonucleotides of the array complementary to JNK1 is ISIS 12539 (JNK1ASISIS12539) (5′-CTCTCTGTAGGCCCGCTTGG-3′) located in the 3′ end of the coding region. Treatment with this oligonucleotide leads to a reduction of steady state mRNA level by 95% (Fig. 8A) as observed 24 hours after a 4 hour exposure of the cells to 0.4 μM antisense oligonucleotides. ISIS 12560 (JNK2ASISIS12560) (5′-GTCCGGGCCAGGCCAAAGTC-3′), located in the 5′ end of the coding region of JNK2 genes, was the most efficient oligonucleotide in reducing JNK2 steady state mRNA levels. Lipofection with this oligonucleotide lead to a decrease in the steady-state JNK2 message level by 92% compared to the control JNK2 steady state mRNA level. The oligonucleotides targeted to the regions flanking either side of the sequences for JNK1ASISIS12539 and JNK2ASISIS12560 also inhibited JNK1 and JNK2 steady state mRNA but in a manner that decreased with increasing distance from the optimum sequence (Fig. 8A and B) suggesting that discrete regions of the target mRNA are accessible and sensitive to the antisense-mediated elimination of steady-state mRNA as previously described (Crooke, 1992, 1998).

Cross inhibition tests demonstrated isoform class specificity. As shown in Fig. 9, JNK1ASISIS12539 eliminates JNK1 mRNA and protein but does not affect JNK2 mRNA and protein and, conversely, JNK2ASISIS12560 has no effect on JNK1 mRNA and protein but abolishes JNK2 mRNA and protein. In the northern analysis we describe an example where two other antisense oligonucleotides from the initial gene screening are less efficient in reducing mRNA steady-state levels of their respective target gene (Fig. 9). Similarly, western analysis shows that lipofections with the antisense oligonucleotides leads to complete elimination of their respective target steady state protein levels, whereas the scrambled sequence versions of the candidate oligonucleotides, JNK1Scr or JNK2Scr have no effect (Fig. 10). Thus effective and specific reagents can be obtained.

These reagents have been used to examine the growth promoting roles of the Jun Kinase pathway in human T98G glioblastoma cells (Potapova *et al.*, 1998), human A549 NSCLC cells (Bost *et al.*, 1997, 1999), and human PC3 prostate carcinoma cells (Yang *et al.*, 1998) and shown to specifically inhibit growth and, in the case of A549 cells, anchorage independent growth. Moreover, these reagents have been used in systemic treatment of established PC3 xenografts and shown to block growth and promote regression of the established tumors in high frequency (Bost *et al.*, 1998). In this case, growth inhibition by antisense treatment of 78% was superior to that of cisplatin treatment alone (47%), however the effects of combined antisense JNK-cisplatin of 89% inhibition was greater than either alone suggesting that, indeed, it may be possible to sensitize solid tumors to cisplatin by elimination of Jun Kinase. It will be of interest, therefore, to determine whether JNK in involved in a general mechanism utilized in other tumor cell lines and/or mediates DNA damage repair of other DNA-damaging agents.

4. CONCLUSIONS

These results have clinical implications with regard to the application of therapies designed to alleviate drug resistance. As tumors progress, particularly if they have been exposed to DNA damaging therapies, they may upregulate their DNA repair response

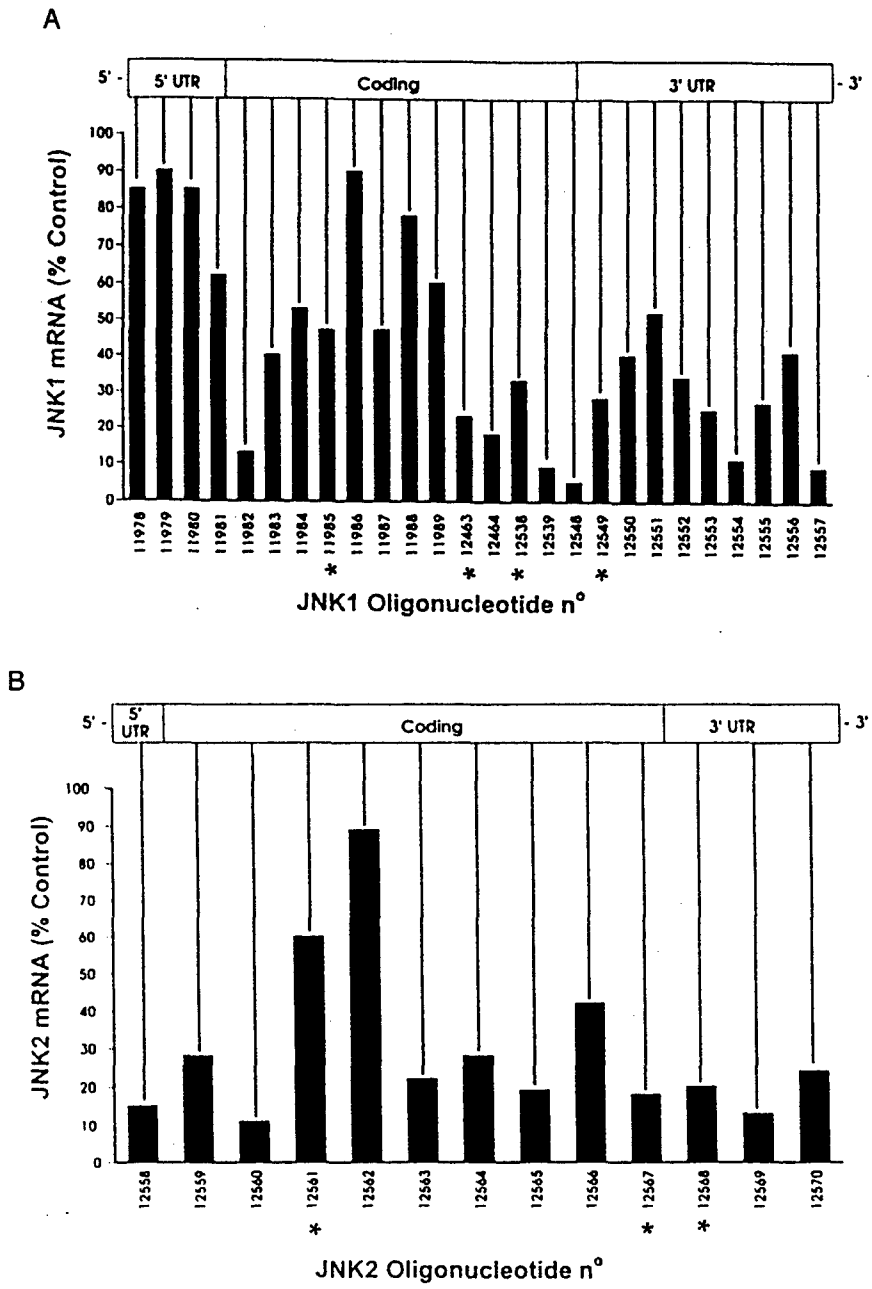

Figure 8. "Messenger walk" survey for the elimination of JNK1 and JNK2 mRNA levels following treatment with phosphorothioate antisense oligonucleotides targeted to JNK1 or JNK2 mRNA. A, JNK1 mRNA steady state levels in A549 cells following treatment with 26 different phosphorothioate antisense oligonucleotides targeted to JNK1 mRNA. JNK1AS^{ISIS12539} gave the most consistent results for the elimination of JNK1 mRNA steady state levels. B, Similar experiment to the previous one (Fig. 1A) was performed with 13 phosphorothioate antisense oligonucleotides complementary to the indicated regions of the JNK2 mRNA. JNK2AS^{ISIS12560} yielded the most consistent results for the elimination of JNK2 mRNA steady state levels. All oligonucleotides are arrayed relative to their complementary sequence along the JNK transcript. The asterisks indicate the oligonucleotides having a very similar nucleotide composition (2–4 bases) to the leading compound. These screenings have been repeated two times with similar results.

Figure 9. A549 cells were treated with 3 different antisense oligonucleotides complementary to JNK1 mRNA (including the active antisense oligonucleotide JNK1AS^{ISIS12539} and 3 different antisense oligonucleotides complementary to JNK2 mRNA (including the active antisense oligonucleotide JNK2AS^{ISIS12560}). 24 hours after a 4 hours transfection with 0.4 μM antisense oligonucleotide, mRNA was prepared and examined by northern analysis, the same membrane was hybridized successively with JNK1 probe, JNK2 probe and the G3PDH probe.

(see Reed, 1998 for review), which may in part involve AP-1 regulated DNA synthesis and repair genes. Elevated c-fos expression, one component of the AP-1 transcription factor, correlates with cisplatin resistance (Scanlon *et al.*, 1991; Funato *et al.*, 1992) and may directly affect the synthesis of AP-1 regulated DNA synthesis and repair genes, such as polymerase β, topoisomerase I and thymidylate synthetase (Scanlon and Kashini-Sabet, 1998). Loss of DNA damage-induced apoptosis through loss of wild-type p53 expression also correlates with drug resistance (O'Connor *et al.*, 1997) and represents an independent mechanism through which tumors acquire resistance to therapy. Because the success or failure of DNA repair could be critical in determining how a cancer cell responds to expression of p53, it may be necessary, in optimizing the benefits of

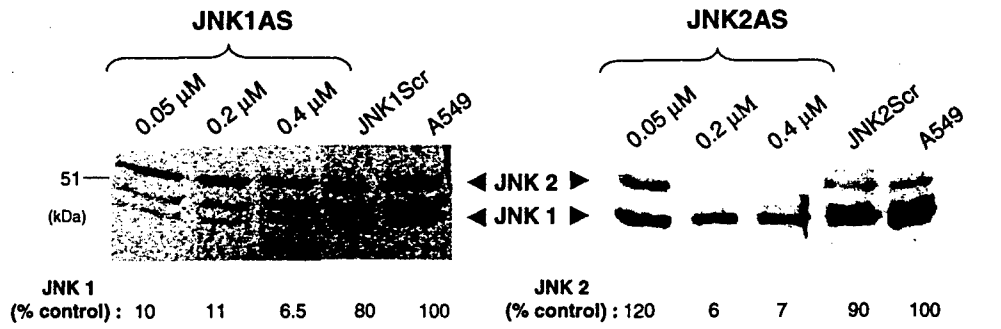

Figure 10. A549 cells were treated with the indicated concentration of phosphorothioate 2′-O-methoxyethyl-modified antisense oligonucleotides JNK1AS^{ISIS15346} or JNK2AS^{ISIS15353} and 0.4 μM of their respective control oligonucleotides JNK1Scr^{ISIS18076} and JNK2Scr^{ISIS18078}. Cell extracts were prepared 36 hours after the transfection and examined by western analysis using anti-JNK1 antibodies (SC-571). Protein level (below) was determined by comparison to respective protein level in untreated cells using the Electrophoresis Documentation and analysis System 120 (Kodak Digital Science™).

p53-based approaches, to concurrently down-regulate the cellular stress and DNA damage response pathways such as the Jun Kinase pathway. Combination approaches targeting independently these two cellular responses to DNA damage can be foreseen today, and may provide an optimal strategy for reversing or alleviating drug resistance in advanced cancers.

ACKNOWLEDGMENTS

The authors would like to acknowledge financial support form the following: grants CA69546 (RAG), CA56834 (DM), CA76173 (DM) from the National Cancer Institute, grant DAMD17-96-1-6038 from the U.S. Army Breast Cancer Research Program (RAG), grant 1PF0140 from the California Cancer Research Program (RAG), grant 83401 from the California Breast Cancer Research Program (DM), and Introgen Therapeutics, Inc. (RAG), The authors would also like to thank Drs. Fred Bost and Olga Potapova for making available to us manuscripts prior to publication. We thank Deborah Wilson, Ph.D. of Introgen Therapeutics, Inc. for providing us with replication recombinant adenoviruses (Ad-p53, Ad-βgal, Ad-Luc). We also thank R. McKay, Ph.D. and N. Dean, Ph.D. of ISIS Pharmaceuticals for their support and materials for the studies reviewed here.

REFERENCES

Badie, B., Kramar, M.H., Lau, R., Boothman, D.A., Economou, J.S., and Black, K.L., 1998, Adenovirus-mediated p53 gene delivery potentiates the radiation-induced growth inhibition of experimental brain tumors, *J. Neurooncol.* **37**:217–222.

Bakalkin, G., Selivanova, G., Yakovleva, T., Kiseleva, E., Kashuba, E., Magnusson, K.P., Szekely, L., Klein, G., Terenus, L., and Wiman, K.G., 1995, p53 binds single stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain, *Nucl. Acids Res.* **23**:362–269.

Barret, J.M., and Hill, B.T., 1998, DNA repair mechanisms associated with cellular resistance to antitumor drugs: potential novel targets, *Anticancer Drugs* **9**:105–123.

Binetruy, B., Smeal, T., and Karin, M., 1991, Ha-ras augments c-Jun activity and stimulates phosphorylation of its activation domain, *Nature* **351**:122–127.

Cavigelli, M., Dolfi, F., Claret, F.X., and Karin, M., 1995, Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation, *EMBO J.* **14**:5957–5964.

Chelliah, J., and Jarvis, W.D., 1996, Effect of 1-beta-D-arabinofuranosylcytosine on apoptosis and differentiation in human monocytic leukemia cells (U937) expressing a c-Jun dominant-negative mutant protein (TAM67), *Cell Growth Differ.* **7**:603–613.

Clarke, A.R., Purdie, C.A., and Harrison, D.J., *et al.*, 1993, Thymocyte apoptosis induced by p53-dependent and independent pathways, *Nature* **362**:849–852.

Crooke, S.T., 1992, Therapeutic applications of oligonucleotides, *Annu. Rev. Pharmacol. Toxicol.* **32**:329–376.

Crooke, S.T., 1998, Molecular mechanisms of antisense drugs: Rnase H, *Antisense Nucleic Acid Drug Dev.* **8**:133–134.

Crul, M., Schellens, J.H., Beijnen, J.H., and Maliepaard, M., 1997, Cisplatin resistance and DNA repair, *Cancer Treat Rev.* **23**:341–366.

Dean, N.M., McKay, R., Miraglia, L., Geiger, T., Muller, M., Fabbro, D., and Bennett, C.F., 1996, Antisense oligonucleotides as inhibitors of signal transduction: development from research tools to therapeutic agents, *Biochem. Soc. Trans.* **24**:623–629.

De Luca, L.G., Johnson, D.R., Whitley, M.Z., Collins, T., and Pober, J.S., 1994, cAMP and tumor necrosis factor competitively regulated transcriptional activation through a nuclear factor binding to the cAMP-responsive element/activating transcription fact element of the endothelial leukocyte adhesion molecule-1 (E-selectin) promoter, *J. Biol. Chem.* **269**:19193–19196.

Derijard, B., Hibi, M., Wu, I.H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R.J., 1994, JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain, *Cell* **76**:1025–1037.

Dorigo, O., Turla, S.T., Lebedeva, S., and Gjerset, R.A., 1998, Sensitization of rat glioblastoma multiforme to cisplatin *in vivo* following restoration of wild-type p53 function, *J. Neurosurg.* **88**:535–540.

Fink, D., Aebi, S., Howell, S.B., 1998, The role of DNA mismatch repair in drug resistance, *Clin. Cancer Res.* **4**:1–6.

Fujiwara, T., Grimm, E.A., Mukhopadhyay, T., Zhang, W.W., Owen-Schaub, L.B., and Roth, J.A., 1994, Induction of chemosensitivity in human lung cancer cells *in vivo* by adenovirus-mediated transfer of the wild-type p53 gene, *Cancer Res.* **54**:2287–2291.

Funato, T., Yoshida, E., Jiao, L., Tone, T., Kashani-Sabet, M., and Scanlon, K.J., 1992, The utility of an anti-fos ribozyme in reversing cisplatin resistance in human carcinomas, *Adv. Enzyme Regul.* **32**:195–209.

Gjerset, R.A., Turla, S.T., Sobol, R.E., Scalise, J.J., Mercola, D., Collins, H., Hopkins, and P.J., 1995, Use of wild-type p53 to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. *Molec. Carcinog.* **14**:275–285.

Gjerset, R.A., Dorigo, O., and Maung, V., *et al.*, 1997, Tumor regression *in vivo* following p53 combination therapy, *Cancer Gene Therapy* **4**:0–2.

Grant, S., Freemerman, A.J., Birrer, M.J., Martin, H.A., Turner, A.J., Szabo, E., and Reed, E., 1998, Platinum-DNA adduct, nucelotide excision repair and platinum based anti-cancer chemotherapy, *Cancer Treat Rev.* **24**:331–344.

Gupta, S., Campbell, D., Derijard, B., and Davis, R.J., 1995, Transcription factor ATF2 regulation by the JNK signal transduction pathway, *Science* **267**:389–393.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M., 1993, Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain, *Genes Dev.* **7**:2135–2148.

Jennerwein, M.M., and Eastman, A., 1991, A polymerase chain reaction-based method to detect cisplatin adducts in specific genes, *Nucleic Acids Res.* **19**:6209–6214.

Lee, S., Elenbas, B., Levine, A., and Griffith, J., 1995, p53 and its 14kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches, *Cell* **81**:1013–1021.

Levine, A.J., 1993, The tumor suppressor genes, *Annu. Rev. Biochem.* **62**:623–651.

Levine, A.J., 1997, p53, the cellular gatekeeper for growth and division, *Cell* **88**:323–331.

Livingstone, C., Patel, G., and Jones, N., 1995, ATF-2 contains a phosphorylation-dependent transcriptional activation domain, *EMBO J.* **14**:1785–1797.

Lotem, J., and Sachs, L., 1993, Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents, *Blood* **82**:1092–1096.

Lowe, S.W., Ruley, H.E., and Jacks, T., and Housman, D.E., 1993, p53-mediated apoptosis modulates the cytotoxicity of anti-cancer agents, *Cell* **74**:957–967.

Lowe, S.W., Bodis, S., McClatchy A., Remington, L., Ruley, H.E., Fisher, D.E., Housman, D.E., and Jacks, T., 1994, p53 status and the efficacy of cancer therapy *in vivo*, *Science* **266**:807–810.

McKay, R.A., Miraglia, L.J., Cummins, L.L., Owens, S.R., Sasmor, H., and Dean, N.M., Characterization of a potent and specific class of antisense oligonucleotide inhibitor of human protein kinase C-alpha expression, *J. Biol. Chem.* **274**:1715–1722.

Miyake, H., Hara, I., Gohji, K., Yamanaka, K., Arakawa, S., and Kamidono, S., 1998, Enhancement of chemosensitivity in human bladder cancer cells by adenoviral-mediated p53 gene transfer, *Anticancer Res.* **18**:3087–3092.

Newell, C.L., Deisseroth, A.B., and Lopez-Berestein, G., 1994, Interaction of nuclear proteins with an AP-1/CRE-like promoter sequence in the human TNF-alpha gene, *J. Leukocyte Biol.* **56**:27–35.

Nguyen, D.M., Spitz, F.R., Yen, N., Cristiano, R.J., and Roth, J.A., 1996, Gene therapy for lung cancer: enhancement of tumor suppression by a combination of sequential systemic cisplatin and adenovirus-mediated p53 gene transfer, *J. Thorac. Cardiovasc. Surg.* **112**:1372–1376, discussion 1376–1377.

Nielsen, L.L., Lipari, P., Dell, J., Gurnani, M., and Hajian, G., 1998, Adenovirus-mediated p53 gene therapy and paclitaxel have synergistic efficacy in models of human head and neck, ovarian, prostate, and breast cancer, *Clin. Cancer Res.* **4**:835–846.

O'Connor, P.M., Jackman, J., Bae, I., Myers, T.G., Fan, S., Mutoh, M., Scuderio, D.A., Monks, A., Sausville, E.A., Weinstein, J.N., Friend, S., Fornace, A.J., Jr., and Kohn, K.W., 1997, Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents, *Cancer Res.*, **57**:4285–4300.

Oshita, F., and Saijo, N., 1994, Rapid polymerase chain reaction assay to detect variation in the extent of gene-specific damage between cisplatin- or VP-16-resistant and sensitive lung cancer cell lines, *Jpn J. Cancer Res.* **85**:669–673.

Potapova, O., Haghighi, A., Bost, F., Liu, C., Birrer, M.J., Gjerset, R., and Mercola, D., 1997, The JNK/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin, *J. Biol. Chem.* **272**:14041–14044.

Potapova, O., Bost, F., Dean, N., and Mercola, D., 1998, Inhibition of the JNK pathway blocks cell cycle progression in human tumor cells, *Proc. AACR*, 89th Annual Meeting, abstract #1720, p. 251.

Reed, E., 1998, Platinum-DNA adduct, nucleotide excision repair and platinum based anti-cancer chemotherapy, *Cancer Treatment Reviews* **24:** 331–344.

Roth, J.A., Swisher, S.G., Merritt, J.A., Lawrence, D.D., Kemp, B.L., Carrasco, C.H., El-Naggar, A.K., Fossella, F.V., Glisson, B.S., Hong, W.K., Khurl, F.R., Kurie, J.M., Nesbitt, J.C., Pisters, K., Putnam, J.B., Schrump, D.S., Shin, D.M., and Walsh, G.L., 1998, Gene therapy for non-small cell lung cancer: a preliminary report of a phase I trial of adenoviral p53 gene replacement, *Semin. Oncol.* **25**:33–37.

Saves, I., and Masson, J.M., 1998, Mechanisms of resistance to xenobiotics in human therapy, *Cell Mol Life Sci.* **54**:405–426.

Scanlon, K.J., Kashani-Sabet, M., 1988, Elevated expression of thymidylate synthase cycle genes in cisplatin-resistant human ovarian carcinoma A2780 cells, *Proc. Natl. Acad. Sci. USA.* **85**:650–653.

Scanlon, K.J., Kashani-Sabet, M., Miyachi, H., Sowers, L.C., and Rossi, J., 1989, Molecular basis of cisplatin resistance in human carcinomas: model systems and patients, *Anticancer Res.* **9**:1301–1312.

Scanlon, K.J., Jiao, L., Funato, T., Wang, W., Tone, T., Rossi, J.J., and Kashani-Sabet, M., 1991, Ribozyme-mediated cleavage of c-fos mRNA reduces gene expression of DNA synthesis enzymes and metallothionein, *Proc. Natl. Acad. Sci. USA.* **88**:10591–10595.

Smeal, T., Binetruy, B., Mercola, D., Birrer, M., and Karin, M., 1991, Oncogenic and transcriptional cooperation with Ha-ras requires phosphorylation of c-Jun on serines 63 and 73, *Nature* **354**:494–496.

Smeal, T., Binetruy, B., Mercola, D., Grover-Bardwick, A., Heidecker, G., Rapp, U.R., and Karin, M., 1992, Oncoprotein-mediated signalling cascade stimulates c-Jun activity by phosphorylation of serines 63 and 73, *Mol. Cell. Biol.* **12**:3507–3513.

Stark, G.R., 1993, Regulation and mechanisms of mammalian gene amplification, *Adv. Cancer Res.* **61**:87–113.

Stein, C.A., and Kreig, A.M., 1994, Problems in interpretation of data derived from *in vitro* and *in vivo* use of antisence oligodeoxynucleotides, *Antisense Res. Dev.* **4**:67–69.

Van Dam, H., Wilhelm, D., Herr, I., Steffen, A., Herrlich, P., and Angel, P., 1995, ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents, *EMBO J.* **14**:1798–1811.

Wilhelm, D., van Dam, H., Herr, I., Baumann, B., Herrlich, P., and Angel, P., 1995, Both ATP-2 and c-Jun are phosphorylated by stress-activated protein kinases in response to UV irradiation, *Immunobiology* **193**:143–148.

Preferential Platination of an Activated Cellular Promoter by *cis*-Diamminedichloroplatinum

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Biochemistry®

Reprinted from
Volume 38, Number 38, Pages 12432–12438

Biochemistry **1999,** *38,* 12432−12438

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Received May 11, 1999; Revised Manuscript Received July 6, 1999

ABSTRACT: This study examines how accessibility to cisplatin on various genomic regions in T47D breast cancer cells, including the retinoic acid receptor β gene promoter and coding region and the dihydrofolate reductase gene promoter and coding region, is affected by treatment of the cells with 9-*cis* retinoic acid, a treatment that activates the retinoic acid receptor β gene promoter in these cells. A PCR-based assay was used to measure cisplatin adduct density based on the inhibition of PCR amplification of templates from cisplatin treated versus untreated cells. Treatment of cells with 9-*cis* retinoic acid enhanced accessibility to cisplatin on the retinoic acid receptor β gene promoter region, but not on the coding regions of that gene nor on the dihydrofolate reductase gene promoter or coding regions, where accessibilities to cisplatin remained 2−4 times lower than on the activated retinoic acid receptor β gene promoter. Examination of smaller regions within this promoter region showed a repression of platination in the 500 bp region surrounding the TATA box in cells prior to 9-*cis* retinoic acid treatment, which was abolished following promoter activation. Differences in sequence composition between the various regions could not fully account for differences in platination, suggesting that structural features such as bends in retinoic acid receptor β gene promoter DNA following gene activation, create energetically favorable sites for platination, and contribute to the cytotoxicity of the drug.

cis-Diamminedichloroplatinum (cisplatin) is a highly effective chemotherapeutic agent used in the treatment of a variety of human tumors (*1*). Its cytotoxicity derives from its ability to damage DNA, forming primarily bifunctional 1,2-intrastrand cross-links between adjacent guanines or adenine-guanine dinucleotides, as well as other minor adducts including interstrand cross-links (*2*). Adduct formation impedes DNA polymerase progression, inhibits DNA synthesis, and triggers apoptosis (*3*−*5*). While inhibition of DNA synthesis is believed to be a critical step in cisplatin's cytotoxicity, it does not by itself account for the marked antitumor efficacy of cisplatin. For example, adducts formed by the geometric isomer of cisplatin, *trans*-diamminedichloroplatinum (transplatin), also impede DNA polymerase progression and inhibit DNA synthesis (*6*). Yet transplatin does not induce apoptosis and has little antitumor activity. Furthermore, while cisplatin causes slowing of DNA synthesis, cells treated with cisplatin do not arrest in S phase, but progress and block in G2 (*7*). In addition, the extent to which DNA synthesis is slowed by cisplatin in sensitive versus resistant cells does not correlate with its relative toxicity to those cells (*8*). Several studies have pointed to transcription as a key target for cisplatin and have suggested that cisplatin's ability to target certain genetic regulatory elements and inhibit specific gene expression may contribute greatly to its toxicity (*9*−*12*).

A structural distortion in DNA occurs upon cisplatin binding that has been proposed to underlie the biological toxicity of cisplatin. Crystallographic studies reveal that the 1,2-cisplatin cross-link induces a bend of about 45° toward the major groove of the DNA helix accompanied by thermal destabilization (*13*). Such a bend appears to generate a structural motif with biological specificity, as certain chromosomal proteins bind with high affinity to these sites. These proteins include histone H1 (*14*), HMG1 (*15*), HMG2 (*16*), the human structure-specific recognition protein SSRP1(*17*, *18*), and the human transcription factor hUBF (*18*), all of which are proteins known to bend DNA and whose binding to DNA might be facilitated by the bends generated by cisplatin. In contrast, the structural effects of transplatin appear to be of greater variability (*19*), and transplatin adducts do not constitute high affinity binding sites for the above-mentioned chromosomal proteins (see ref *20* for review). Cisplatin's toxicity may therefore be related to the structural consequences of adduct formation, which could involve a disruption of normal binding of chromosomal proteins, or an impediment to conformational changes that are necessary for biological regulation.

In the studies reported here we have addressed the possibility that cisplatin may actually target chromosomal regions such as transcriptional promoters, where DNA bends or partial unwinding of DNA may occur following transcription factor recruitment to an activated promoter. If so, such a structural motif may provide a preferential target for cisplatin and contribute to the cytotoxicity of this drug. We have examined cisplatin adduct formation on promoter and

† This work was supported in part by grants (to R.A.G.) from the National Cancer Institute CA69546 and from the U.S. Army Breast Cancer Research Program DAMD17-96-1-6038.

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10.1021/bi991079r CCC: $18.00
Published on Web 08/26/1999

downstream regions of the retinoic acid receptor β (RARβ)[1] gene in T47D breast cancer cells, where this gene undergoes retinoic-acid-dependent activation. Adduct formation as determined by a quantitative PCR-based assay was observed to be significantly higher on the activated RARβ promoter than on a downstream region of the same gene, as well as on the coding and promoter regions of the constitutively expressed housekeeping gene, dihydrofolate reductase (DHFR). Adduct formation on the DHFR promoter, which does not undergo retinoic-acid-dependent activation, did not increase following retinoic acid treatment of cells. Cisplatin's cytotoxicity may therefore derive in part from its ability to target and disrupt the function of certain genetic regulatory loci such as the RARβ promoter.

EXPERIMENTAL PROCEDURES

Cell Culture. T47D breast cancer cells were purchased from ATCC and maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 50 μg/mL gentamycin. Cell culture reagents were purchased from Irvine Scientific, Santa Ana, CA. All experiments were performed using charcoal-treated serum (600 mg of activated charcoal/50 mL of serum for 10 min at 4 °C, followed by filtration).

Analysis of RARβ and DHFR Gene Expression. About 5 × 10^5 T47D cells (at about 70% confluency) were treated as indicated and total cellular RNA was prepared using the Rneasy kit from Qiagen and following the manufacturer's procedure. One microgram of RNA was reverse transcribed into cDNA in a 20 μL reaction containing 0.5 mM dNTPs (Pharmacia), 100 μg/mL oligo dT (Promega), 2 units of RNAsin (Promega), 10 units of Moloney Murine leukemia virus reverse transcriptase (Promega), and reverse transcriptase buffer (Promega). This cDNA was then used as a template for quantitative PCR. Primers were chosen so as to amplify a 178 base region of the RARβ coding sequence encompassing parts of exons 4 and 5, as well as a 246 base region of the coding sequence of the dihydrofolate reductase from exons 1−4. The sequences are as follows: RARβ message (forward), 5′-GTGTACAAACCCTGCTTCGTCT-GC-3′; (reverse) 5′-CTGGAGTCGACAGTATTGGCATCG-3′; DHFR message (forward), 5′-GGTTCGCTAAACTG-CATCGTCGC-3′; (reverse) 5′-GTGGAGGTTCCTTGAG-TTCTCTG − 3′.

Amplification conditions were as described below for the PCR-stop assay. Quantitative conditions were verified by preparing cDNA with 100 and 10 ng of RNA (in addition to 1 μg of RNA) and amplifying serial 2-fold dilutions of the cDNA from 2 to 0.25 μL of template to show that product formation was proportional to input template under our conditions. Following amplification, products were analyzed by agarose gel electrophoresis followed by band quantitation using a Kodak digital camera.

Cell Viability. Following treatments, cell viability was monitored by adding 10% Trypan blue and determining the fraction of cells that excluded the dye.

Cisplatin Treatments. Cells were plated at 50% confluency in six-well plates in medium supplemented with 10% charcoal-treated FBS in addition to the other standard additives described above. Following attachment, cultures were pretreated 24 h in the presence or absence of 1 μM 9-*cis* retinoic acid (Sigma, St. Louis, MO), followed by a 2 h incubation in the presence or absence of 1 mM cisplatin (Platinol, aqueous solution at 1 mg/mL, purchased from local pharmacies). The preincubation medium was then replaced, and genomic DNA was prepared 24 h later as described below. Although DNA repair has been observed to occur over 24 h in cells treated with lower doses of cisplatin (*21*−*22*), at the high levels used in our studies we observed an overall increase in adduct formation over 24 h, so that adduct densities approached 1−4 adducts/10 Kb and were therefore readily detectable in our assay. The increase in adduct formation over time suggests that residual cisplatin continued to cause DNA damage and that repair did not keep pace with adduct formation under our conditions.

Preparation of Genomic DNA. DNA was prepared from treated cultures using the QIAmp blood kit essentially following the manufacturer's protocol, except that cells were lysed directly on the plate in the presence of PBS, Qiagen protease, and lysis buffer supplied in the kit. Following purification, DNA was adjusted to 0.25 mg/mL in sterile water and stored at −20° C until use.

Analyses of DNA Damage by PCR Stop Assay. Quantitative PCR was used to compare cisplatin adduct formation on specific regions of DNA. The assay, known as the PCR stop assay, has been described (*23*). Because Taq polymerase is blocked at cisplatin adducts, the relative efficiency of PCR amplification of genomic DNA from cisplatin-treated versus control cells decreases in proportion to platination levels. The relative PCR efficiency is equivalent to the frequency (P) of undamaged strands within a population. P is related to the average number (n) of cisplatin adducts per fragment, by the Poisson formula: $P = e^{-n}$, or $-(\ln P) = n$. Adducts per Kb would then be equal to $-(\ln P)$/(size of fragment in Kbs). We found that fragment sizes of around 1 Kb provided a sufficiently large target size to enable measurement of adduct densities in the range 0.1−0.4 adducts/Kb observed here. A drop in the PCR signal of damaged DNA to 0.67 of the control signal would therefore reflect an average cisplatin adduct density of $-(\ln 0.67) = 0.4$ adducts/fragment. Standard deviations among triplicate PCRs were in the range of ±5%, meaning that adduct densities of less than about 0.05 adducts/fragment were undetectable. For each primer pair, we verified that product formation was directly proportional to input template DNA by performing a pilot experiment with serial 2-fold dilutions of template, followed by electrophoresis on a 1% agarose gel containing 0.5 μg/mL ethidium bromide. Bands were quantitated using Kodak digital camera and analysis software. Depending on the primer set, the amount of template used in the PCR reaction ranged 0.03−0.25 μg/25 μL reaction. Reactions were performed in 25 μL containing template DNA, 25 pmol each of forward and reverse primer, 250 μM dNTPs (Pharmacia), 1.25 units of Taq polymerase (Qiagen), 1× buffer (Qiagen), and solution Q (Qiagen). The amplification program was as follows: 1 cycle (94 °C, 1 min 30 s); 25 cycles (94 °C, 1 min; 57 °C, 1 min; 70 °C, 2 min, 30 s); 1 cycle (94 °C, 1 min; 57 °C, 1 min; 70 °C, 7 min). Two independent templates

[1] Abbreviations: RARβ, retinoic acid receptor β; RARE, retinoic acid response element; PCR, polymerase chain reaction; DHFR, dihydrofolate reductase; ATCC, American type culture collection; PBS, phosphate-buffered saline; Kb, kilobase; bp, base pair; CDS, coding sequence.

Table 1: Primers Used for PCR-stop Assay of Genomic DNA

gene	region amplified	primer sequences (5′→3′)	product size (base pairs)
RAR β	RARβ Promoter (includes A,B,C below)	1: CGAGTGCAGTCAATTCAGCCAGG (for) 2: GCTTATCCTCTAGGTGTGGAGGC (rev)	1043
	(A) promoter, upstream and including TATA and RARE	1: see above (for) 3: CTTCCTACTACTTCTGTCACACAG (rev)	624
	(B) promoter region of TATA and RARE	4: GGGAGAGAAGTTGGTGCTCAACG (for) 5: CCTTCCGAATGCGTTCCGGATC (rev)	483
	(C) promoter, downstream and including TATA and RARE	6: GCTTTTGCAGGGCTGCTGGGAG (for) 2: see above (rev)	592
	RARβ CDS (1036 bp) (exon 10)	7: GGTGCAGAGCGTGTAATTACCTTG(for) 8: CTGCCTTGGAGGCTATCATTACTG(rev)	1036
	RARβ CDS (483 bp) (exon 10)	7: see above (for) 9: GGTCTTTGCCATGCATCTTGAGTG (rev)	483
DHFR	DHFR promoter	10: CAGAATGGGAGTCAGGAGACCTG (for)	1000
	11: GCAGAAATCAGCAACTGGGCCTC (rev)		
	DHFR CDS (1062 bp) (exons 1,2,adjacent introns)	12: CCGTAGACTGGAAGAATCGGCTC (for) 13: CAGTTGCCAATTCTGCCCCATGC (rev)	1062
	DHFR CDS (472 bp) (exon 1 and adjacent introns)	14: CAATTTCGCGCCAAACTTGACCG (for) 15: GAGCTCTAAGGCACCTGACAAAC (rev)	472
	DHFR CDS (272 bp) (exon 1 and adjacent introns) (internal control)	16: GGTTCGCTAAACTGCATCGTCGC (for) 17: CAGAAATCAGCAACTGGGCCTCC (rev)	272

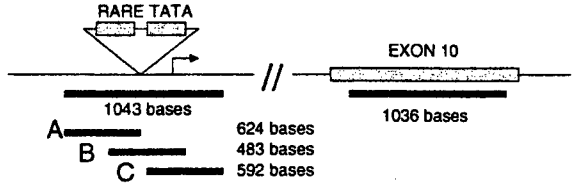

FIGURE 1: Location and sizes of PCR amplification products from the RARβ gene of T47D cells (see also Table 1). For the analysis in Figure 3, PCR primers were chosen so as to amplify a 1043 bp region of the RARβ promoter, including the retinoic acid response element (RARE) and TATA box, and a 1036 bp region of the RARβ coding sequence (CDS). For the analysis in Figure 4, PCR primers were chosen so as to amplify the subregions A, B, and C.

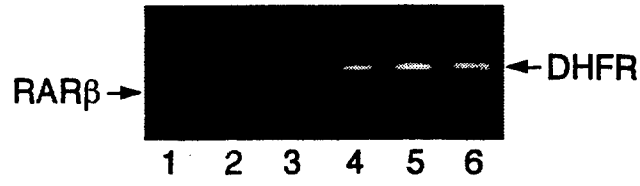

FIGURE 2: Quantitative RT-PCR analysis of RARβ gene expression (lanes 1−3) and DHFR gene expression (lanes 4−6) in T47D cells under various conditions. Lanes 1 and 4: untreated T47D cells. Lanes 2 and 5: T47D cells treated 24 h with 1 μM 9-*cis* retinoic acid. Lanes 3 and 6: cells treated 24 h with 9-*cis* retinoic acid, followed by 2 h with 200 μM cisplatin. Quantitative conditions for cDNA synthesis and PCR amplification were verified as described in the Experimental Procedures. A total of 10 and 2 μL, respectively, of the RARβ and DHFR PCR reactions were analyzed.

were prepared for each treatment condition, and each one was analyzed in triplicate. As an internal PCR control for each template, a 270 bp fragment of the dihydrofolate reductase gene was amplified. This fragment is too small to register significant levels of damage under the conditions we used, and its amplification product was seen to vary by less that 5% among the various templates. The primers 1−17 used for PCR amplification of various regions of genomic DNA are summarized in Table 1. Figure 1 shows a schematic representation of regions amplified from the RARβ gene.

RESULTS

Transcriptional Response of the RARβ Gene to 9-cis Retinoic Acid and Cisplatin. Figure 2 shows the results of an RT-PCR assay demonstrating retinoic-acid-dependent activation of the RARβ gene (lane 2 compared to lane 1) and cisplatin-mediated inhibition of this activation (lane 3). Under the same conditions, we observed little change in DHFR expression (lanes 4−6), indicating that expression of this gene is not retinoic acid dependent and that cisplatin has little immediate effect on its expression. This is consistent with another study in which treatment with cisplatin did not alter levels of DHFR message (*24*), although long term in vitro selection for cisplatin-resistant ovarian carcinoma cell lines by repeated exposure to cisplatin has been reported to generate variants that overexpress DHFR (*25*). Genomic DNA from cells before and after treatment with 9-*cis* retinoic acid was then used to analyze platination of the RARβ gene in its active and inactive state.

Preferential Cisplatin Adduct Formation on the Activated RARβ Gene Promoter. We performed a PCR stop assay to examine cisplatin adduct density on genomic DNA templates from 9-*cis*-retinoic-acid-treated (RA+) or -untreated (RA−) cells. Regions spanning about 1 Kb in length of the RARβ promoter (1043 bp), the RARβ coding sequence (1036 bp), the DHFR promoter (1000 bp), and the DHFR coding sequence (1062 bp) were examined as defined by primers listed in Table 1. PCR products were analyzed on agarose gels (Figure 3A) and bands were quantitated in order to determine the relative PCR efficiencies from platinated versus unplatinated templates. Using templates from (RA−) cells (Figure 3A, lanes 1 and 2), the relative PCR efficiencies from platinated versus unplatinated templates (lane 2 versus lane 1) were as follows: RARβ promoter (0.88), RARβ coding sequence (0.85), DHFR promoter (0.85), DHFR coding sequence (0.87). Using templates from (RA+) cells (lanes 3 and 4), the relative PCR efficiencies from platinated versus unplatinated templates (lane 4 versus lane 3) were as follows: RARβ promoter (0.65), RARβ coding sequence (0.88), DHFR promoter (0.9), DHFR coding sequence (0.88). On the basis of the PCR results, the cisplatin adducts densities were calculated by the Poisson equation described above. The results plotted in Figure 3B represent the averages

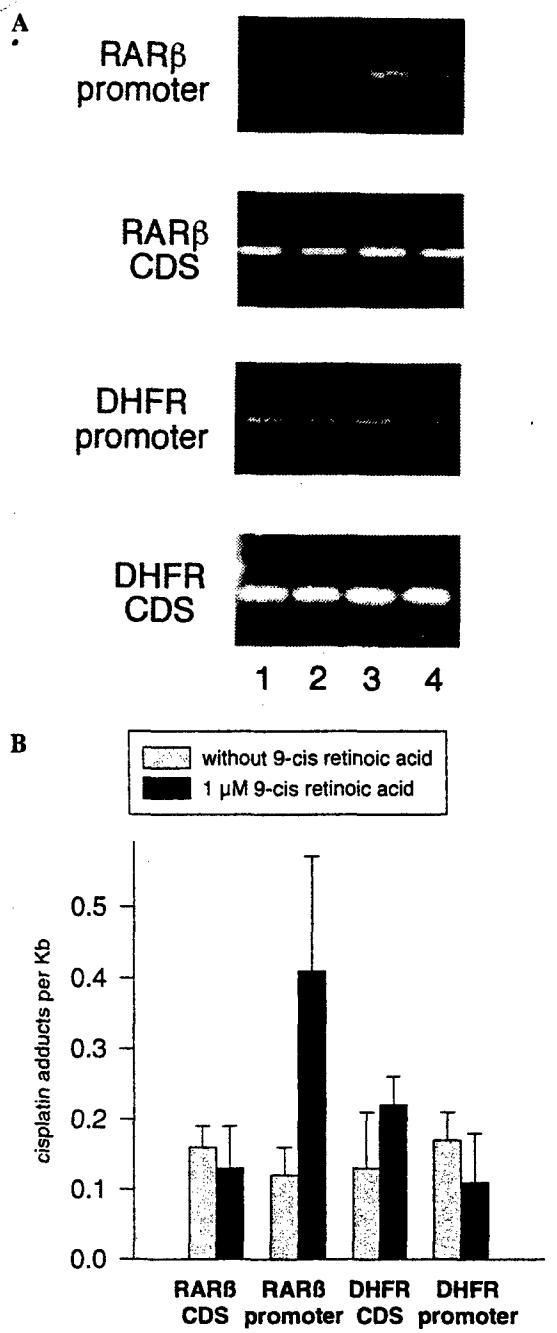

FIGURE 3: Cisplatin adduct formation on various regions of T47D cell genomic DNA as determined by the PCR stop assay. Regions analyzed were the RARβ CDS (1036 bp), the RARβ promoter (1043 bp), the DHFR CDS (1062 bp), and the DHFR promoter (1000 bp). Genomic DNA was prepared from cells pretreated in the presence (RA+) or absence (RA−) of 9-*cis* retinoic acid for 24 h followed by 2 h in the presence or absence of 1 mM cisplatin. (A) Agarose gel analysis of PCR products from one experiment. Lanes 1 and 2 (RA− cells). Lanes 3 and 4 (RA+ cells). Lanes 1 and 3 (no cisplatin). Lanes 2 and 4 (plus cisplatin). (B) Bar graph showing cisplatin adducts per Kb as calculated from the PCR results as described in the Experimental Procedures. The results represent the averages and standard deviations of two separate experiments with independently prepared templates, with each experiment being performed in triplicate. For each experiment, results were corrected for variations in the 270 bp PCR product of the DHFR gene.

and standard deviations of two separate experiments with independently prepared templates, with each experiment being performed in triplicate and corrected for variations in the 270 bp PCR product of the DHFR gene.

As shown in Figure 3B, in the case of the RARβ CDS (1036 bp), the DHFR CDS (1062 bp), and the DHFR promoter (1000 bp), treatment of cells with 9-*cis* retinoic acid has little effect on cisplatin adduct density. Thus, adduct densities (in adducts per Kb) on these regions in untreated cells (RA−) are observed to be 0.16 ± 0.03, 0.13 ± 0.08, and 0.17 ± 0.04, respectively. Adduct densities on these same regions in 9-*cis*-retinoic-acid-treated cells (RA+) are observed to be 0.13 ± 0.06, 0.2 ± 0.04, and 0.11 ± 0.07, respectively.

In contrast, cisplatin adduct density on the RARβ promoter (1043 bp) is enhanced in cells following treatment of cells with 9-*cis* retinoic acid (Figure 3B). For this region, adduct densities increase from 0.12 ± 0.04 adducts/Kb in untreated cells (RA−) to 0.4 ± 0.16 adducts/Kb in treated cells (RA+). Cisplatin adduct density measured on the active RARβ promoter region in RA+ cells is therefore about 2 times as high as on the DHFR CDS (1062 bp) or DHFR promoter regions in RA+ cells, and nearly 3.5 times as high as on the inactive RARβ promoter in RA− cells. Cisplatin adduct density on the constitutively expressed DHFR promoter does not increase following 9-*cis* retinoic acid treatment (Figure 3B).

To determine the extent to which differences in sequence composition might account for differences in adduct density, we examined the number of GG and AG dinucleotide pairs (the major sites of adduct formation in DNA) in the various DNA regions studied, as summarized in Table 2 (column 2). Clusters of three or four Gs were scored as one site. The number of potential sites for adduct formation was in turn used to estimate the relative frequencies of cisplatin targets per Kb compared to the DHFR CDS (1062 bp) region (Table 2, column 5), given that AG is targeted by cisplatin only about 40% as frequently as GG dinucleotides (*2*). We find that cisplatin adducts form on the RARβ promoter (1043 bp) about two times as frequently as we would anticipate on the basis of sequence composition alone. That is, the estimated frequency of targets is about the same for the RARβ promoter (1043 bp) region and the DHFR CDS (1062 bp) region, yet the observed adduct frequency on the promoter region is about two times what it is on the DHFR CDS (1062 bp) region (0.4 ± 0 0.16 versus 0.2 ± 0.04 adducts/Kb). Similarly, the estimated frequency of targets on the RARβ promoter (1043 bp) is about two times the estimated frequency on RARβ CDS (1036 bp), yet the observed adduct frequency is three times greater on the promoter compared to the CDS (0.4 ± 0 0.16 versus 0.13 ± 0.06 adducts/Kb). This suggests that other factors also contribute to the preferential targeting of cisplatin to the induced RARβ promoter.

Location of Cisplatin Adducts within the Promoter Region. To further localize adduct formation within the promoter, we subdivided the RARβ promoter (1043 bp) region into three overlapping regions A (624 bp), B (483 bp), and C (592 bp) shown schematically in Figure 1, and defined by primers listed in Table 1. As shown in Figure 4, in the absence of promoter activation with 9-*cis* retinoic acid, adduct formation is virtually undetectable in the central region (B) encompassing the TATA box and RARE (retinoic acid response element) and approximately equivalent in regions A and C in adducts per Kb to what we observe on the larger 1043 base region of the promoter (0.17 adducts/

Table 2: Comparison of Expected versus Observed Adduct Density in Various Gene Fragments from Cells Treated with 9-*cis* Retinoic Acid and 1 mM Cisplatin

1	2	3		4	5	6	7
gene fragment	no. of GG and AG sites	cisplatin targets per fragment[a] (a)	(b) sum	targets/Kb; column3(b) ÷ fragment length (Kb)	targets/Kb relative to DHFR; column 4 ÷ 83	expected adducts per Kb based on observed DHFR[b]; column 5 x (0.2)	observed adducts per Kb[c]
DHFR CDS	GG 77	GG 77	88	83	1.0	0.2	0.2 ± 0.04
1062 bases	AG 26	AG 11					
RARβ CDS	GG 25	GG 25	47	45	0.54	0.11	0.13 ± 0.06
(1036 bp)	AG 56	AG 22					
RARβ promoter	GG 72	GG 72	92	88	1.1	0.21	0.40 ± 0.16
(1043 bp)	AG 50	AG 20					
DHFR promoter	GG 84	GG 84	106	106	1.3	0.26	0.11 ± 0.07
(1000 bp)	AG 55	AG 22					

[a] AG adducts are about 40% as likely to form as GG adducts (*16*); therefore, AG targets (column 3a) = AG sites (column 2) × 0.4. [b] From column 7. [c] Calculated from PCR data in Table 2 using Poisson equation relating adducts per fragment to PCR signal, and correcting for fragment size.

Kb on each of regions A, C versus 0.12 adducts/Kb on the larger fragment overall). This suggests that some portion of the central region of the promoter included in fragment B is protected from platination in the uninduced state.

Following promoter activation, all three regions A, B, and C sustain elevated levels of damage, (0.34 ± 0.07, 0.43 ± 0.07, and 0.27 ± 0.11 adducts/Kb, respectively), which is on the average what we observe on the larger 1043 base region of the activated promoter (0.4 ± 0.16 adducts/Kb). This confirms the measurement of adduct density on the larger 1043 base region (Figure 3 and Table 2) and supports the conclusion drawn from the comparison of sequence composition that the activated RARβ promoter sustains levels of cisplatin damage greater than would be expected on the basis of base composition alone. Because regions A, B, and C do not differ significantly between themselves in GG and AG sequence compositions, the result here suggests that cisplatin adducts are distributed evenly over the activated promoter and that protection from platination in region B is abolished by promoter activation.

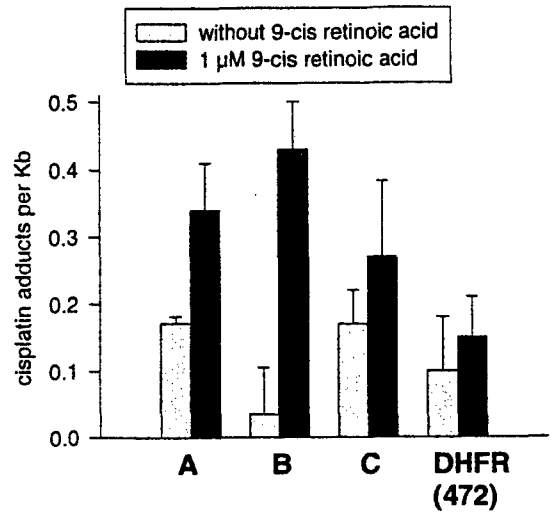

FIGURE 4: Cisplatin adduct formation on three overlapping regions A (624 bp), B (483 bp), and C (592 bp) of the RARβ promoter 1043 bp region shown schematically in Figure 1. Primers used are listed in Table 1. Cells were treated as in Figure 3. Results represent the average of triplicate assays.

DISCUSSION

In these studies, we have used a PCR-based assay to detect platination on genomic regions encompassing about 1 Kb of the RARβ promoter, the RARβ coding sequence (CDS), the DHFR promoter, and the DHFR CDS in T47D breast cancer cells. We see that treatment of these cells with 9-*cis* retinoic acid, a treatment that activates the RARβ promoter but not the DHFR promoter, results in a 3-fold increase in accessibility of the RARβ promoter region to cisplatin (0.4 ± 0.16 versus 0.12 ± 0.04 adducts/Kb). In contrast, the accessibilites to cisplatin of the DHFR promoter, DHFR CDS, and RARβ CDS are not significantly altered by treatment of cells with 9-*cis* retinoic acid, and observed adduct densities remain about 2−4-fold lower than on the activated RARβ promoter (0.11 ± 0.07, 0.2 ± 0.04, and 0.13 ± 0.06 adducts/Kb, respectively). The activated RARβ promoter appears therefore to be hypersensitive to cisplatin adduct formation. Preferential platination of the RARβ promoter relative to the other regions examined could be attributed only in part to differences in sequence composition, suggesting that structural factors also play a role in directing adduct formation to certain sites.

The possibility that promoter-specific structural changes influence reactivity with cisplatin is further supported by the analysis of subregions of the larger RARβ promoter fragment, where we see that accessibility to cisplatin increases over the entire promoter region following activation of the promoter. Furthermore, the accessibility to cisplatin of the central region of the promoter, including the TATA box and RARE appears to be suppressed in the inactive state, and this suppression is relieved upon promoter activation. In this case, inhibition of accessibility to cisplatin might be due to steric hindrance due to the possible positioning of a nucleosome at or near the start site of transcription. A recent analysis of a large set of unrelated RNA polymerase II promoters, both TATA-containing and TATA-less, has revealed a common bendability profile of DNA just downstream of the start site of transcription, suggesting that the DNA could wrap around protein in a nucleosome (*26*). In addition, the complementary triplet pair, CAG/CTG, shown to correlate with nucleosome positioning (*27*, *28*) is over-represented in the region just downstream of the transcription start site (*26*). Complexation with nucleosomes near the transcription start site of the inactive promoter might

therefore impede adduct formation by cisplatin, and displacement or destabilization of nucleosomes by the transcription initiation complex might remove this impediment.

Following transcriptional activation, the RARβ promoter not only becomes more accessible to cisplatin than it was prior to transcriptional activation but also appears to bind more cisplatin than would be expected based on sequence composition alone. This suggests that some structural feature of the DNA of the activated promoter might make it an energetically favorable site for cisplatin adduct formation. One such feature might be an induced bend in the promoter DNA generated as a result of protein−protein interactions between transcription factors bound to distant sites (see ref *29* for review). Such a bend might provide the stored energy required for strand separation and initiation of transcription, and this process could be blocked by the formation of a cisplatin adduct.

The retinoids *all-trans*-retinoic acid (ATRA) and 9-*cis* retinoic acid (9-*cis* RA), as well as several synthetic analogues of these natural retinoids, have attracted considerable attention as potential chemotherapeutic agents for the treatment of promyelocytic leukemia (*30*) and other cancers (*31*−*33*). As ligands for nuclear retinoic acid receptors (RARα, β, or γ) and retinoid X receptors (RXRα, β, or γ), retinoids regulate the expression of sets of overlapping genes involved in the regulation of cell proliferation, differentiation, and apoptosis (*34*−*36*). *all-trans*-Retinoic acid, binding to RAR α, β, or γ, and 9-*cis* retinoic acid, binding to either RAR α, β, or γ or RXR α, β, or γ, promote the heterodimerization of these receptors and facilitate the binding of the heterodimeric complex to specific response elements (RAREs) in the promoter regions of retinoic-acid-responsive genes. This binding is believed to facilitate the recruitment of the components of the basal transcription complex to the promoter and promote chromatin remodeling required for the onset of transcription. While these events may by themselves lead to partial suppression of some solid tumors, in most examples studied, retinoids showed more potential when used in combination with DNA-damaging chemotherapies such as cisplatin, etoposide, and 5-fluorouracil (*37*−*39*). A synergistic activity between retinoids and cisplatin has been observed only when retinoid treatment preceded cisplatin treatment, and this correlated with a 1.5-fold increase in cisplatin adduct content of DNA without a change in cisplatin uptake (*39*). These data suggest that retinoid treatment induces an event or events that directly enhance cisplatin cytotoxicity and are consistent with the possibility suggested by our data that retinoids may generate cisplatin-hypersensitive structures that contribute to an enhanced response to cisplatin.

These data implicate chromatin organization as a component influencing the toxicity of cisplatin and add further support to a growing body of evidence pointing to the transcription process as a target for cisplatin mediated cytotoxicity. Cisplatin adduct formation may disrupt transcription through several mechanisms, including the following. (a) Cisplatin adducts may generate inappropriate binding sites for transcription factors and chromosomal proteins with HMG domains, titrating them away from their natural sites (*18*). A variety of HMG-box chromosomal proteins involved in the transcription complex bind to cisplatin adducts (*14*−*18*). (b) Cisplatin may inhibit transcription factor binding to certain promoters, as has been shown for a stably integrated mouse mammary tumor virus promoter-driven reporter gene (*40*). In this case, cisplatin treatment prior to hormonal induction prevented activation of the reporter gene and blocked recruitment of the transcription complex to the promoter. (c) By targeting GG dinucleotides, cisplatin may inhibit certain regulatory elements rich in strings of guanosines, leading to selective inhibition of such promoters (*9*, *10*). (d) As suggested by our results, cisplatin may target a DNA structure generated in the active promoters of certain genes, such as the RARβ gene. Assembled on activated promoters may be some of the factors needed to trigger apoptosis, including the transcription factor p53. Of interest is the recent identification of HMG-1, which is known to bind to cisplatin adducts, as a specific activator of p53 (*41*).

The differential toxicities of various platinum drugs may therefore be related to their respective abilities to mimic or disrupt chromatin structures critical to transcription. Further studies will be required to determine to what extent the observations made in this study extend to other promoters and to other DNA damaging agents.

REFERENCES

1. Loehrer, P. J., and Einhorn, L. H. (1984) *Ann. Intern. Med. 100*, 704−713.
2. Eastman, A. (1986) *Biochemistry 25*, 3912−3915.
3. Pinto, A. L., and Lippard, S. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4616−4619.
4. Howle, J. A., and Gale, G. R. (1970) *Biochem. Pharmacol. 19*, 2757−2762.
5. Chu, G.(1994) *J. Biol. Chem. 269*, 787−790.
6. Heiger-Bernays, W. J., Essigmann, J. M., and Lippard, S. J. (1990). *Biochemistry 29*, 8461−8466.
7. Sorenson, C. M., and Eastman, A. (1988) *Cancer Res. 48*, 4484−4488
8. Sorenson, C. M., and Eastman, A. (1988) *Cancer Res. 48*, 6703−6707.
9. Gralla, J. D., Sasse-Dwight, S., Poljak, L. G. (1987) *Cancer Res. 47*, 5092−5096.
10. Buchanan, R. L., and Gralla, J. D. (1990) *Biochemistry 29*, 3436−3442.
11. Evans, G. L., and Gralla, J. D. (1992) *Biochem. Biophys. Res. Commun. 184*, 1−8.
12. Evans, G. L., and Gralla, J. D. (1992) *Biochem. Pharmacol. 44*, 107−119.
13. Takahara, P. M., Rosenzweig, A. C., Frederick, C. A., and Lippard, S. J. (1995) *Nature 377*, 649−652.
14. Yaneva, J., Leuba, S. H., van Holde, K., and Zlatanova, J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 13448−13451.
15. Pil, P. M., and Lippard, S. J. (1992) *Science 256*, 234−237.
16. Billings, P. C., Davis, R. J., Engelsberg, B. N., Skov, K. A., Hughes, E. N.(1992) *Biochem. Biophys. Res. Commun. 188*, 1286−1294.
17. Bruhn, S. L., Pil, P. M., Essigmann, J. M., Housman, D. E., and Lippard, S. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2307−2311.
18. Treiber, D. K., Zhai, X., Jantzen, H.-M., and Essigman, J. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5672−5676.
19. Bellon, S. F., Coleman, J. H., and Lippard, S. J. (1991) *Biochemistry 30*, 8026−8035
20. Zlatanova, J., Yaneva, J., and Leuba, S. H. (1998) *FASEB J. 12*, 791−799.
21. Bohr, V. A. (1991) *Carcinogenesis 12*, 1983−1992.
22. Rampino, N. J., and Bohr, V. A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10977−10981.
23. Jennerwein, M. M., and Eastman, A. (1991) *Nucleic Acids Res. 19*, 6209−6214.
24. Sheibani, N., and Eastman, A. (1990) *Cancer Lett. 52*, 179−185.

25. Scanlon, K. J., and Kashani-Sabet, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 650−653.
26. Pedersen, A. G., Baldi, P., and Chauvin, Y. (1998) *J. Mol. Biol. 281*, 663−673.
27. Baldi, P., Brunak, S., Cauvin, Y., and Krogh, A. (1996) *J. Mol. Biol. 263*, 503−510.
28. Liu, K., and Stein, A. (1997) *J. Mol. Biol. 270*, 559−573.
29. Van der Vliet, P. C., and Verrijzer, C. P. (1993) *Bioessays 15*, 25−32.
30. Huang, M. E., Ye, Y. C., and Chen, S. R. (1988) *Blood 72*, 567−572
31. Redfern, C. P., Lovat, P. E., Malcolm, A. J., and Person, A. D. (1995) *Eur. J. Cancer 31A*, 486−494.
32. Anzano, M. A., Byers, S. W., Smith, J. M., Peer, C. W., Mullen, L. T., Brown, C. C., Roberts, A. B., and Sporn, M. B. (1994) *Cancer Res. 54*, 4614−4617.
33. Gottardis, M. M., Lamph, W. W., Shalinsky, D. R., Wellstein, A., and Heyman, R. A. (1996) *Breast Cancer Res. Treat. 38*, 85−96.
34. Breitman, T., Selonic, S., and Collins, S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2936−2940.
35. Sporn, M., and Robert, A. (1984) *J. Natl. Cancer Inst. 73*, 1382−1387.
36. Martin, S., Bradley, J., and Cotter, T. (1990) *Clin. Exp. Immunol. 79*, 448−453.
37. Sacks, P. G., Harris, D., and Chou, T.-C. (1995) *Int. J. Cancer 61*, 409−415.
38. Guchelaar, H. J., Timmer-Bosscha, H., Dam-Meiring, A., Uges, D. R., Oosterhuis, J. W., de Vries, E. G., and Mulder, N. H. (1993) *Int. J. Cancer 55*, 442−447.
39. Caliaro, M. J., Vitaux, P., Lafon, C., Lochon, I., Nehme, A., Valette, A., Canal, P., Bugat, R., and Jozan, S. (1997) *Br. J. Cancer 75*, 333−340.
40. Mymryk, J. S., Zaniewski, E., and Archer, T. K. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2076−2080.
41. Jayaraman, L., Moorthy, N. C., Murthy, K. G., Manley, J. L., Bustin, M., and Prives, C. (1998) *Genes Dev. 12*, 462−472.

BI991079R

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 26 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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